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Thesis

DEVELOPMENT OF A HYBRID VASCULAR BYPASS GRAFT USING A TISSUE ENGINEERING APPROACH

Submitted for the Degree of Doctor of Philosophy (University of London)

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List of Abbreviations

AFM	Atomic Force Microscopy
ANOVA	Analysis Of Variance
ATR	Attenuated Total Reflection
bFGF	Basic Fibroblast Growth Factor
bpm	Beats Per Minute
BTEC	Biomaterials and Tissue Engineering Centre
CA	Cell attachment
CABG	Coronary Artery Bypass Graft
CHD	Coronary Heart Disease
CPU	Compliant Poly(carbonate-urea)Urethane
CVD	Cerebrovascular Disease
Dacron	Polyethylene Terephthalate
DMEM	Dulbecco's Modified Eagle Medium
EC	Endothelial Cell
ECM	Extracellular Matrix
EPC	Endothelial Progenitor Cells
FAPP	Fibronectin Adhesion Promoting Peptide
FEPP	Fibronectin Engineered Polymer Protein
FEPP+	Fibronectin Engineered Polymer Protein Plus
FN	Fibronectin
FTIR	Fourier Transform Infra-Red Spectroscopy
GFP	Green Fluorescent Protein
H&E	Haematoxylin and Eosin
hVSMC	Human Vascular Smooth Muscle Cells

HUCV	Human Umbilical Cord Vein
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	Intercellular Adhesion Molecule
In	Indium
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PGA	Polyglycolic Acid
PHA	Polyhydroxyalkanoate
PTFE	Polytetrafluoroethylene
PVD	Peripheral Vascular Disease
RGD	Arginine-Glycine-Aspartate
RGDS	Arginine-Glycine-Aspartate-Serine
RLG	Radioluminograph
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SIS	Small Intestine Submucosa
SMC	Smooth Muscle Cells
SV	Saphenous Vein
SVC	Superior Vena Cava
TE	Tissue Engineering
TEBV	Tissue-Engineered Blood Vessel
tPA	Tissue plasminogen activator
UC	Umbilical Cord
VCAM	Vascular Cellular Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor

XPS	X-ray Photo Spectrometry

Abstract

Introduction: A third of patients needing arterial bypass grafts lack sufficient autologous vessels. Prosthetic alternatives – principally PTFE and Dacron – have poor patency rates because of compliance mismatch with elastic arteries and inherent surface thrombogenicity. The aim of this research was to develop –for the first time - a hybrid tissue-engineered bypass graft consisting of an elastic scaffold of compliant poly(carbonate-urea)urethane (CPU), incorporated with human smooth muscle cells (SMC) and endothelial cells(EC).

Methods: 1) Methods of human vascular SMC and EC extraction were assessed for both saphenous vein and umbilical cord vessels. 2) Extracted cells were assessed by immunostaining and for SMC the ability to contract collagen gels. 3) Coating CPU with various biomolecules (to make the surface bioactive), cell seeding density and attachment period were assessed for their impact on SMC attachment. 4) Cell growth on CPU was investigated by retroviral transduction of the GFP (Green Fluorescent Protein) gene and assays of cell viability and nucleic acid content. 5) A bioreactor and pulsatile flow circuit was developed for long-term culture of cells on CPU. 6) The impact of shear stress pre-conditioning on cell retention on the hybrid bypass graft was investigated under arterial flow conditions.

Results: 1) SMCs were reliably extracted from umbilical cord and saphenous vein. ECs were only reliably extracted from umbilical cord. 2) Cord SMCs grew faster than saphenous vein SMCs (doubling time of 3.4 ± 0.6 days against 5.6 ± 1.9 days; $p = 0.0227$): all SMCs stained for alpha-actin and contracted collagen gels; 3) SMC attachment to CPU was significantly enhanced by Fibronectin-like Engineered Polymer Protein Plus [FEPP+] (from $20.7\pm4.6\%$ to $31.5\pm5.9\%$: $p<0.01$), higher cell density but not longer attachment period. 4) Transducing SMCs with GFP

successfully allowed live cell imaging on CPU and assays of both viable cells and nucleic acid confirmed cell growth on CPU. 5) The flow circuit successfully allowed long-term sterile culture of cells on CPU. 6) Retention of SMCs and ECs on CPU was improved by a period of shear stress pre-conditioning: from 56.7±7.0% to 76.2±6.5% SMC retention and from 45.6±2.3% to 67.4±4.0% EC retention ($p<0.03$).

Conclusion: Pre-lining CPU with FEPP+ enhances SMC attachment. GFP-transduction allows study of SMC growth on CPU. Pre-conditioning enhances retention of SMCs and ECs onto CPU, probably because the mechanical stimuli orientate the cells and increase the release of matrix proteins and attachment factors. The stage is now set for developing a hybrid graft for *in vivo* studies.

Dedication

I would like to dedicate this thesis to my family, especially my son who was born during this period.

I would also like to specifically thank my father for his endless encouragement and support.

Statement of Originality & Acknowledgements

I would like to confirm that I was responsible for all the work in this thesis but had help with specific areas. All assistance was from other members of the University Department of Surgery except where stated otherwise.

Matthew Button taught me the techniques for smooth muscle cell extraction, culture and how to form collagen gels. Furthermore Matthew Button gave valuable contributions to the development of the flow circuit.

Geoff Punshon taught me the techniques for endothelial cell extraction, culture and how to use the assays for both Alamar blue and Pico Green.

Alok Tiwari taught me how to perform immunohistochemistry.

Henryk Salacinski helped with the research on attachment factors and the technique for radiolabelling cells.

The bioreactors have a long history in the department with contributions to their development from Alex Seifalian, Karen Cheetham and Graeme Barden amongst others. More recent refinements which critically allowed them to achieve long-term sterility were from Matthew Button and myself.

Calculations of shear stress were performed by Professor Alexander Seifalian using an ultrasound device with a wall tracking system.

Geoffrey Boxer and Uzma Qureshi from the Department of Oncology, Royal Free and University Medical School, taught me the technique of Phosphoimaging as well as the analysis of images. They also helped with the staining of slides.

Mel Tohill and Giorgio Terenghi, formerly of the Blond McIndoe Centre within the University Department of Surgery, provided laboratory facilities and taught me the technique for the transduction of green fluorescent protein.

Francis Moll from the Department of Pathology prepared the photomicrographs of immunohistochemically stained smooth muscle cells.

Innes Clatworthy from the Department of Electron Microscopy within the Department of Pathology was responsible for all the SEM pictures.

I also thank Freemedic Ltd for funding my research for one year and Credent Ltd who supplied the graft material.

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Finally, this thesis would not have been possible without the help and advice of my co-supervisors: Professors George Hamilton, Barry Fuller and Alexander Seifalian.

Hypothesis

Previous work at the Biomaterial and Tissue Engineering Centre had developed a graft material with attractive mechanical characteristics for arterial bypass grafting. Compliant poly(carbonate-urea)urethane (CPU) has a 0.9mm thick honeycomb structure that allows it to maintain compliance similar to that of human artery and pulsatile flow *in vivo* through a mechanism of wall compression that accommodates increases in volume without the need for external dilation – similar to the action of a sponge. Previous studies have shown that in addition to improved compliance, polyurethanes have better thromboresistance, with rapid ingrowth of living tissue through the graft and reduced anastomotic hyperplasia. CPU has been shown to have superior ability to attach cells compared to traditional graft materials like Dacron.

The hypothesis under study is whether a hybrid graft using scaffold, cells and extracellular matrix can be developed as a potential graft for small diameter arterial bypass. I hypothesise that the use of a compliant poly(carbonate-urea)urethane scaffold will enhance the feasibility of such an approach.

Chapter 1

THE PROBLEM OF OCCLUSIVE ARTERIAL DISEASE

1.1 Introduction

Atherosclerosis is a disease process of the large and medium-sized arteries characterised by thickening and loss of elasticity of arterial walls due to deposition of yellowish plaques of cholesterol, lipoid material and lipophages in the intima and inner media(1). From a clinical standpoint it principally manifests itself in three main spheres: coronary heart disease (CHD), cerebrovascular disease (CVD) and peripheral vascular disease (PVD).

<table>
<tr><th>Disease</th><th>Manifestation</th><th>Anatomical Area</th><th>Clinical Area</th><th>Medical Rx</th><th>Radiological Rx</th><th>Surgical Rx</th></tr>
<tr><td>CHD</td><td>Angina & Myocardial Infarction</td><td>Coronary circulation</td><td>Cardiology Cardiac Surgery</td><td rowspan="4">Risk Factor Reduction Pharmacotherapy</td><td>Angioplasty & stenting</td><td>Bypass grafting</td></tr>
<tr><td rowspan="2">PVD</td><td>Ischaemic limbs</td><td>Iliac circulation & distal</td><td rowspan="2">Radiology Vascular Surgery</td><td rowspan="2">Stenting controversial: undergoing clinical trials</td><td>Insertion of graft</td></tr>
<tr><td>Aneurysm</td><td>Principally aorta</td><td>End-arterectomy</td></tr>
<tr><td>CVD</td><td>Stroke</td><td>Carotid Circulation</td><td>Neurology Radiology Vascular Surgery</td><td></td></tr>
</table>

Table 1.1: Manifestations & Treatment of Atherosclerosis Throughout the Body

As can be seen from Table 1.1, no matter where the atherosclerosis is, the first treatments involve Risk Factor Reduction and Pharmacotherapy (Table 1.2).

RISK FACTOR	NON-DRUG METHODS	PHARMACOTHERAPY
Genetic		
Male		HRT for women
Sedentary lifestyle	Exercise(2)	
Obesity	Diet Exercise	Appetite suppressants: sibutramine Reduction of fat absorption: orlistat
Smoking	Cessation(3)	Nicotine patches(4)
Hypertension	Lifestyle change(5;6) (diet, exercise, weight loss)	B-blockers Thiazide diuretics Angiotensin II blockers ACE inhibitors(7;8)
Hyperlipidaemia	Diet	Fibrates & Statins(9)
Diabetes	Diet	Sulphonylureas, Biguanides, Glitazones, Insulin(10;11)
Hyperhomocystinaemia		Folic Acid +/-Vitamin B supplementation(12)
Pro-thrombotic tendency		Anti-platelets(13)

Table 1.2: Atherosclerotic Risk Factors and Treatments

For both ischaemia of the coronary and distal limb circulations, interventions can be either radiological in the form of angioplasty and stenting or surgical bypass grafting.

For CHD the indications for radiological intervention are somewhat complex and increasing in number. Essentially angioplasty is felt to be the optimum treatment in the immediate aftermath of myocardial infarction(14;15) and also for those suffering from persistent angina and with a stenosis amenable to treatment(16;17). Bypass surgery is preferred principally for those patients with lesions of the main stem of the left coronary artery or lesions affecting at least three of the main branches of the left or right coronary artery(18-23). For PVD, significant claudication causing either severe limitations to lifestyle or critical ischaemia resulting in either rest pain or tissue loss requires intervention in the form of angioplasty or stenting. When this is not possible or has failed surgical bypass remains the only option unless it is felt that amputation of the limb is more appropriate(24).

1.2 Conduits for bypass surgery

1.2.1 Autologous Vessels

The best conduits for bypass surgery are the patient's own blood vessels. For the coronary circulation these are the Internal Mammary Arteries which have patency rates of 90% at 10 years(25). Unfortunately, though both arteries can be used, there is often a need for more than two vessels to be bypassed at any one time. Therefore other vessels are needed. This usually means the Long Saphenous Vein which has a patency rate of 50% at 10 years(26;27). Other options include the Radial, Gastroepiploic and Inferior Epigastric Arteries, Short Saphenous, Basilic and Cephalic Veins(28-32).

For the peripheral circulation venous bypasses are mostly used, though a few examples using the radial artery have been reported(33;34) These can either be in the *in situ* or reversed vein position. The *in situ* technique affords anatomical matching

between anastomosis sites but requires ablation of valves with a valvulotome, with probable damage to the endothelium. Reversing the vein escapes the trauma of a valvulotome but the anastomotic sites will not be matched anatomically. In actual fact the patency rates are equivalent no matter how the vein is inserted(35).

Unfortunately about a third of patients will have insufficient autologous vessels and in these cases there is no option except to use alternative grafts(36).

1.2.2 Human Allografts and Bovine Heterograft

Possible non-autologous sources of biological grafts include Human Umbilical Cord Vein (HUCV), cryopreserved saphenous vein and bovine heterografts(37-39). Unfortunately, these are complicated by early aneurysmal formation and / or suffer from poor patency and so are not used in the UK(40), although there is some argument for their use in infected fields where autologous vessels are unavailable and prosthetic vessels are contra-indicated(39;41;42).

1.2.3 Prosthetic Vessels

As mentioned above a third of patients lack a suitable vein. Therefore surgeons have turned to prosthetic vessels. Foremost amongst these are Dacron (polyethylene terephthalate) and PTFE (polytetrafluoroethylene). In the United Kingdom there is preference for PTFE, though trials suggest there is no significant difference between the two(43-47). In bypasses involving large diameter vessels with good flow rates, as in the above knee femoral-popliteal position, these prosthetic vessels give good patency rates comparable to vein(48-50). However, there is no doubt that patency is worse where the anastomosed vessel is less than 6mm in diameter as in the coronary circulation, and also where the flow rates are low, as in the infrapopliteal circulation(49-55). The patency of prosthetic grafts is improved by the use of an interposition vein collar or patch to attach the prosthesis to the artery(56-60).

1.3 Reasons for Graft Failure

The cause of graft occlusion can be divided in to early, mid-term and late(61). Early failure occurring within 30 days of surgery tends to be due to technical problems, poor inflow and/or outflow and acute thrombosis caused by activation of the clotting cascade - related to Virchow's classic triad of coagulability of the blood, vessel wall damage and stasis of the blood(62). Mid-term from 3 months to 2 years is due to narrowing of the lumen of the graft, principally around the distal anastomosis due to 'neointimal hyperplasia'(63;64). Late occlusion after 2 years is due to the underlying atherosclerotic degeneration.

Neointimal hyperplasia consists principally of a proliferation of vascular smooth muscle cells associated with synthesis of extracellular matrix(63;65;66). The exact cause of this is not truly understood. Risk factors for this include disturbed flow(67), damage to the vessel wall(65) and compliance mismatch between the elastic artery and the relatively inelastic prosthetic graft(68). This mismatch occurs both along the graft length and the anastomosis due to non-compliant suture material and suturing technique(69). The difference in compliance results in haemodynamic changes including cyclic stress, turbulent flow and flow separations, which culminates in the release of growth factors which stimulate neointimal hyperplasia. The benefit of interposition vein collars(70) is partly related to their superior compliance because of the improved pulsatile flow profile propagation.

Ultimately the area of neointimal hyperplasia consists of an excess of SMCs in the intimal area with associated ECM. The source of these SMCs is a matter for some debate(71). Historically, they were believed to be derived from the arterial media from where they migrate and proliferate(72-76). More recently animal experiments

have suggested that in fact they may be derived from a stem or progenitor cell population circulating in the blood but originating from the bone marrow(77-80).

The other major cause for the failure of prosthetic grafts is their inherent thrombogenicity.

1.3.1 Reducing Graft Thrombogenicity

Approaches include lining or bonding with anticoagulant chemicals like heparin(81;82) or lining with endothelial cells (EC) in a process known as seeding.

A) Anticoagulant Chemicals

Anticoagulant chemicals have the problem of having a finite lifespan and so only work in the short term. However, recent animal studies have shown some promise with improved patency in canine models by immobilising heparin onto PTFE grafts(81). Furthermore heparin-bonded Dacron showed superior patency to PTFE in a clinical study of above and below-knee arterial bypasses(82;83).

B) Seeding

ECs are capable of manufacturing antithrombotic chemicals themselves and inhibiting SMC proliferation - and so offer a long-term solution to graft thrombogenicity and neointimal hyperplasia. The difficulty with seeding is getting the ECs in the first place and then getting them to remain on the graft.

Sources for ECs(84) include vein(85-93), fat(94-106), blood(107) and even bone marrow(108-110). Methods to improve cell attachment onto the graft surface include the use of chemical coatings, pre-clotting, chemical bonding and surface modifications. This whole subject has been recently reviewed comprehensively by Salacinski et al(111).

Two methods for putting the ECs on the graft have been identified: single and two-stage seeding. Single-stage seeding(112) involves extraction and seeding at the same

time as implanting the graft in the patient. Whilst representing an ideal from a clinician's viewpoint, trials have not proven this method to be of any benefit(89;113). The reasons for this include the fact that it is difficult to get sufficient numbers of cells in the first place to cover the graft surface and then on exposure to arterial pressures and flow most of the ECs are simply washed away(114-118).

In comparison, two-stage seeding has shown itself to markedly improve the patency of prosthetic grafts to levels only seen in vein bypasses(119;120). This method involves initial extraction of the ECs from vein, followed by a culture period to generate sufficient ECs to achieve supra-confluent levels of ECs on the graft. The graft is then implanted into the patient at a separate operation. The problem with this method is that it requires two operations and a waiting period of a month or so for culturing of the ECs(87;121). This is not an option for emergency bypass, which is the main indication in peripheral vascular disease as there is little indication for doing such a demanding procedure for intermittent claudication(24;122).

1.3.2 Improving Graft Compliance

Another area of research would be to improve upon the poor compliance of PTFE and Dacron. There has indeed been little in the way of successful materials research in the last few decades to provide an alternative prosthetic material except for research into polyurethanes.

A) Polyurethanes

The Biomaterials and Tissue Engineering Centre (BTEC) at the Royal Free & University College Medical School in association with industry has been looking into polyurethanes. This has resulted in a compliant poly(carbonate-urea)urethane (CPU) which is now a commercial product for haemodialysis access and is undergoing trials

as a bypass graft for peripheral vascular surgery. This material offers several potential key advantages over PTFE and Dacron including superior compliance, tissue and blood compatibility. Clinical studies have shown that in addition to improved compliance, polyurethanes have better thromboresistance, with rapid ingrowth of living tissue through the graft and reduced anastomotic hyperplasia(123-126). As an access conduit for haemodialysis, when compared to PTFE, polyurethane grafts are easier to cannulate, seal rapidly and can be used sooner after access surgery has been performed(127-130). Furthermore, polyurethanes require less time for compression haemostasis and do not suffer from persistent post-operative oedema or seroma.

Unfortunately, polyurethane grafts historically do have a significant rate of thrombosis and infection, with variable patency rates in the short and long term: some studies showing inferior patency rates when compared to PTFE grafts(129;131). However, the main problem limiting their use so far has been the occurrence of aneurysm formation due to biodegradation of the soft segments which together with the hard segments make up the chemical structure of polyurethane grafts(132).

In contrast to the aforementioned problems, CPU has undergone *in vitro* degradation tests and has been implanted in a dog model for 36 months, demonstrating very high biostability(133-135). Furthermore the CPU has a 0.9mm thick honeycomb structure that allows it to maintain compliance similar to that of human artery(136) and pulsatile flow *in vivo* through a mechanism of wall compression that accommodates increases in volume without the need for external dilation – similar to the action of a sponge.

CPU is manufactured using a new type of stress free process and is comprised of an inner and outer skin and a spongy middle that permits transmission of pulsatile flow(137). Importantly, this compliance remains even after perigraft scar tissue

formation. Furthermore, this structure allows a graft to be developed which can mimic the range of compliances of the host artery, which change with age, disease states and across different vascular systems. Because CPU can be modified to match the unique mechanical properties of the required application, the compliance of the final version of the CPU graft was selected based on measured ranges of compliance of aged and diseased lower limb arteries(138). The compliance of the CPU graft has been compared to artery, vein, Dacron and ePTFE over mean arterial pressure (MAP) of 30-100mmHg(139). The compliance of the CPU graft was similar to artery at mean pressures of 30-60mmHg whilst it was significantly more compliant than ePTFE and Dacron at MAP of 30-100mmHg and 30-90mmHg respectively.

CPU has also been shown to have superior ability to attach EC, which can be further improved by pre-lining the graft with attachment factors like collagen and fibronectin(118;136;139;140). Recent work has shown the possibility of bonding peptides and anticoagulants to further enhance EC attachment and to possibly reduce the risk of thrombosis(141).

1.4 Aim of Thesis

The aim of this thesis was to look into sources of cells required for development of a hybrid tissue-engineered graft for use in bypass grafting of small arterial vessels less than 6mm in diameter. The basic constituents of the graft include a compliant scaffold, cells (principally SMC & EC) and extracellular matrix. The various options for each of these components will be investigated and discussed.

Chapter 2

TISSUE ENGINEERING OF BLOOD VESSELS

2.1 Introduction

Tissue engineering (TE) is a multi-disciplinary field combining biology, materials science and surgery to provide living tissue products to restore, maintain or improve tissue function(142). This hopefully will meet the needs for donor organs and tissues, but TE also offers the promise of being able to dramatically expand our ability to repair tissues, improve surgical procedures and thus significantly improve the quality of life.

It is felt that TE would be particularly valuable in the production of vascular grafts. The reason for this is the massive need and precarious supply of these grafts for both coronary artery bypass grafting (CABG) and lower limb bypass grafting for peripheral vascular disease (PVD).

2.2 Tissue Engineering Approaches

There are three main approaches to TE: infusing cells or cell substitutes, the use of tissue-inducing materials and thirdly the implantation of cells seeded in scaffolds.

2.2.1 Infusing isolated cells or cell substitutes.

Nabel and Plautz introduced genetically modified endothelial and smooth muscle cells into discrete segments of denuded pig artery using a double balloon catheter(143-145). They were able to confine this genetic transformation to the target area and avoided unwanted transfection at distant sites. This may be useful after procedures such as angioplasty where there is a high rate of recurrent stenosis due to neointimal hyperplasia – genetically modified cells with anti-thrombotic and anti-proliferative properties would hopefully reduce the risk of restenosis. A variation of this work involved *in situ* transfection of rabbit femoral veins with an adenoviral construct for

the thrombolytic tissue plasminogen activator. These veins were then used as arterial bypasses and showed significant reduction in thrombus formation compared to controls containing the adenoviral construct for beta-galactosidase and veins without transfected virus(146). A review including clinical trials of genetic interventions for vein bypasses offers a more complete study of this exciting area(61).

Furthermore emerging work on stem cells shows that progenitor cells can be infused into the circulation and preferentially settle in areas of ischaemia promoting angiogenesis and new tissue growth(147-151). The main problems with this approach are that cells may not maintain their function *in vivo* for long and immunological reactions to the cells can occur. The latter problem at least can be overcome by using autologous cells as has been suggested in some recent clinical studies, though again long-term outcomes are awaited(152-157). Another source of controversy for these cells is whether or not these cells transdifferentiate into the appropriate local cells or just fuse with them(158;159).

2.2.2 Use of tissue-inducing materials

This includes scaffolds, chemotactic chemicals and growth factors to replace lost tissue or modify dysfunctional tissue from existing local and functional cell populations. This approach requires the discovery, isolation, purification and mass-production(160) of substances which then need to be delivered to their cellular targets.

An interesting example involves an approach to limiting post-angioplasty neointimal hyperplasia by using perivascular polymeric biospheres containing elastase. These created a chemotactic gradient of elastin degradation which re-directed SMC proliferation from the lumen to the periphery(161). Perivascular implants containing ECs also showed an ability to reduce neointimal thickening: this could not have been

due to cell-cell interactions but must have been a result of the release of cellular products(162-164).

The use of growth factors to improve blood supply to ischaemic tissues in a process called therapeutic angiogenesis has shown promise at the clinical level. Research has focussed on three growth factors: basic fibroblast growth factor (bFGF / FGF-2), vascular endothelial growth factor (VEGF) and hepatocyte growth factor(165-167). For PVD patients the TRAFFIC study showed significantly improved walking distance 90 days after intra-arterial infusion of recombinant FGF-2(168;169), though the RAVE study did not show any benefit in terms of walking distance or quality of life for intramuscular injection of VEGF(170).

2.2.3 Implantation of cells seeded in scaffolds

Thirdly, implantation of cells seeded in scaffolds(142). This approach is the most commonly used method and can be subdivided into open and closed systems. In a closed system the cells are separated from the body by a membrane which permits diffusion of nutrients and wastes but blocks larger elements like immune cells. This system can be implanted or used as an extra-corporeal device. In open systems the cells are attached onto matrices, which can be either natural - like collagen - or synthetic like polytetrafluoroethylene (PTFE). This whole system is then implanted into the body. This is the most popular approach so far. The open approach can further be subdivided by the type of matrix or scaffold used – either natural or synthetic. Synthetic matrixes or scaffolds can then be divided into biodegradable or permanent.

The application of TE to blood vessels should ideally result in the production of conduits with the properties outlined in Table 2.1(171;172).

Biological	Mechanical	Physical	Commercial
Vasoreactive: dilate / constrict to neural & chemical stimuli	Strength to resist bursting	Leak-proof: avoids haemorrhage through its walls	Can be tailored to individual: length diameter etc...
Non-thrombogenic	Avoids kinking even over joints	Porosity for healing and angiogenesis	Cheap to manufacture
Biostable: does not weaken *in vivo* to cause aneurysms & rupture	Hold sutures under circumferential and longitudinal tension		Short time period from request to implantation
Biocompatible: not inflammatory, toxic carcinogenic or immunogenic	Retain axial and radial compliance and pulsatility		
Infection-resistant			

Table 2.1: Ideal Properties of a Tissue-Engineered Blood Vessel

2.3 Anatomy and Physiology of Blood Vessels

There are three concentric layers in a normal blood vessel whether artery or vein. There is an inner *tunica intima* with a monolayer of endothelial cells supported by a basement membrane and a sparse smooth muscle cell (SMC) layer. This layer functions as a barrier as well as having sensory and secretory roles. Around the intima is the *tunica media* containing many more SMCs, collagen and elastin. This layer, especially in arteries, controls blood flow through the vessel by either constricting or dilating. The outer layer is the *tunica adventitia* composed predominantly of fibroblasts(173). The vascular cells are surrounded by the extracellular matrix (ECM), which combines with the cells to provide the biomechanical properties of the vessel. Figure 2.1 shows the layers of the vessel wall diagrammatically.

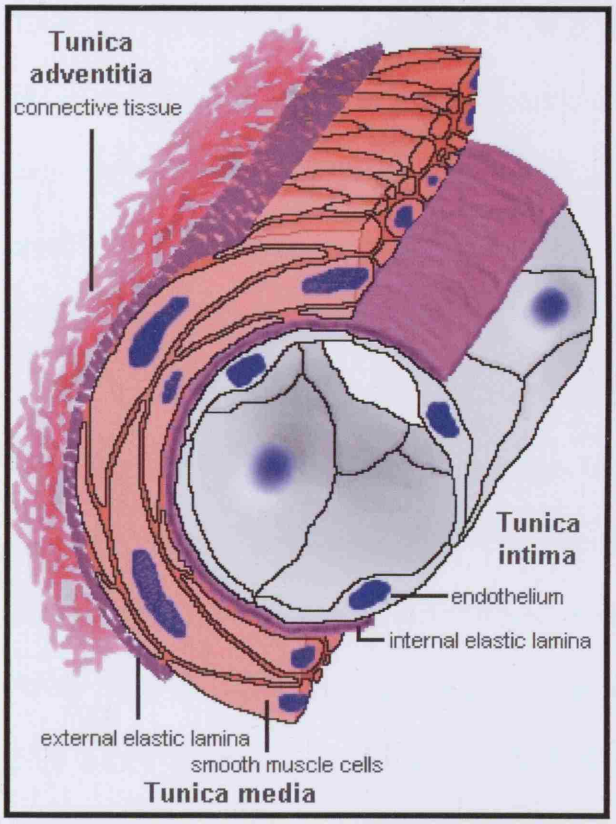

Figure 2.1: Layers of Blood Vessel Wall

The exact proportions of the various constituents vary depending on the type of vessel and where it is situated with respect to the heart. The ECM consists mainly of collagen (especially types I and III), elastin in the form of fibres, proteoglycans, hyaluronan and glycoproteins (like laminin and fibronectin). The collagens provide the tensile stiffness, the elastin the elastic properties, the proteoglycans contribute to compressibility, and combined with the collagen and elastin they are responsible for the viscoelastic properties(173-175).

Replication of this concentrically layered structure has been the basis for the efforts of researchers trying to produce tissue-engineered blood vessels (TEBV).

2.4 Previous Approaches

There are several key components that need to be considered in order to produce a tissue-engineered vascular graft. Scaffolds are used to grow cells and tissues and as such are a critical component of the final graft. In contrast a mandrel essentially confers a physical presence around which cellular and tissue development occurs, but is ultimately removed from the final graft. Extracellular matrix in various preparations can be a component of the scaffold or added to it. Finally, the last elements of variability in tissue engineering are the types of cells used.

However, equally critical to this 'mix' are the signals to which these cells are exposed. The signals which affect the behaviour of these cells are from three sources. Firstly from the fluid flowing through the vessel – *in vivo* this is blood. Next those from the ECM – this is not merely a collection of proteins serving as a biological glue but also a source of regulatory signals. Finally the mechanical environment of the vessel provides signals imposed by the local haemodynamics of the vascular system(176).

2.4.1 Scaffolds

a) Natural

These are scaffolds derived from animal tissue itself. Lantz developed a biologic vascular graft material made from small intestine submucosa (SIS) and tested it in dogs. The graft was prepared by removing a jejunal segment, which had its superficial tunica mucosa, tunica serosa and tunica muscularis externa removed by abrasion. This left tunica submucosa with attached stratum compactum (dense collagen layer) and muscularis mucosa of the tunica mucosa. This material was used as an autograft, allograft and xenograft demonstrating biocompatability and high patency rates (75% overall) in aorta, carotid and femoral arteries as well as the superior vena cava (SVC). The grafts after 90 days were similar to either SVC or artery under histological examination. Furthermore when challenged with a bacterial load they were able to clear the infection much more successfully than ePTFE(177). Similarly, Huyunh constructed a scaffold from a collagen biomaterial derived from the submucosa of the small intestine and type I bovine collagen. The inner lining was treated with heparin and this acellular graft was implanted into rabbit aortas with 100% patency at up to 90 days(178).

Decellularised natural scaffolds have been used by a number of researchers. Bader used porcine aorta decellularised using trypsin, these xenografts were then repopulated with human myofibroblasts and endothelial cells from saphenous vein biopsies(179). Clarke decellularised bovine ureters, using hypotonic water and ribonucleases, which were then grafted into dog aortas with 100% patency and no aneurysms at 10 months(180). Conklin(181) decellularised porcine carotid arteries using detergents and enzymes. These scaffolds were then covalently linked to heparin resulting in reduced *in vitro* thrombogenicity. Furthermore the compliance was

similar to natural vessels with excellent burst and suture-retention strengths. After implantation as xenografts into dog carotids, smooth muscle cells had repopulated the wall and endothelial cells had lined the lumen by two months. Unfortunately the animal studies were too short to draw any significant conclusions. Kaushal used decellularised porcine iliac vessels to seed ovine endothelial progenitor cells (EPC). As a carotid interposition graft EPC-seeded grafts remained patent for 130 days, and once explanted these grafts exhibited contractile activity and nitric-oxide-mediated vascular relaxation that were similar to native carotid arteries. In comparison, non-seeded grafts occluded within 15 days(182).

The obvious advantage of natural scaffolds is that they are composed of extra-cellular matrix proteins typically found in the body. If the scaffold is derived from vessels, the three-dimensional architecture is very similar to that of the vessel it is replacing, thus conferring good mechanical and physical properties. However, whilst the results above are encouraging, there remain concerns over transmission of endogenous retroviruses. A recent study of acellular porcine vessels implanted into sheep showed that porcine retrovirus was not detectable in either recipient blood or explanted vessels six months after surgical implantation – thus providing some reassuring evidence regarding this issue(183). Despite this infective proteins like prions remain a concern even when human vessels are used.

b) Synthetic biodegradable

Synthetic biodegradable scaffolds are not found in nature. Over a period of time they degrade so that they are no longer found within the graft.

Greisler's group have used biodegradable grafts without any added cells or ECM. This approach relies on the scaffold material to induce a replacement vascular tissue

structure before the scaffold itself degrades. 7½ months after woven absorbable polyglycolic acid (PGA) prostheses were implanted into rabbit aortas, the grafts were replaced with smooth-muscle-like myofibroblast media and an endothelialised lumen. However, 11% of these grafts became dilated and 13% had intimal hyperplasia(184). Greisler then used absorbable polydioxanone prostheses, again implanted into rabbits, for up to 12 months. This time only 3.6% (1 of 28) became aneurysmal and again the grafts developed a smooth-muscle-like layer lined with endothelium(185). Finally a composite graft of polypropylene and either polydioxanone and polyglactin was implanted into dogs. As an aortoiliac graft in dogs they achieved 1 year patencies of 86% for the polydioxanone composite and 90% for the polyglactin composite; this compared with 68% and 54% for the non-biodegradable Dacron and ePTFE respectively(186).

Yue used a microporous biodegradable graft from polyurethane-based material and seeded it with rat SMCs. These were then implanted into the rat aorta and demonstrated superior patency compared to non-seeded grafts(187). Niklason pipetted bovine aortic SMCs in suspension onto PGA scaffolds chemically modified by sodium hydroxide. The grafts were then exposed to pulsatile pressure before seeding with ECs. When grafted into swine, the pulsed grafts showed 100% patency at four weeks(188). Shum-Tim used a co-polymer of PGA and polyhydroxyalkanoate (PHA) as a scaffold onto which was seeded a mixture of SMCs, ECs and fibroblasts – cultured as explants from lamb carotid arteries. When grafted into the lamb abdominal aorta these were all patent at 150 days compared to controls which just had the acellular PGA-PHA co-polymer and which all thrombosed(189).

c) Synthetic permanent

These are scaffolds not found in nature which persist indefinitely as part of the graft. Usually they are based on materials already used for bypass grafting and therefore with established clinical track records. In their landmark paper, Weinberg & Bell used Dacron as their scaffold, which was embedded into collagen. SMCs were then cultured in the graft before ECs were seeded onto the inner lining(190). This resulted in a graft which had an *in vitro* burst strength of 323 mmHg. Baguneid used a variation of this, where porcine SMCs were allowed to contract collagen around a Dacron scaffold. It was demonstrated that luminal pre-coating with fibronectin and one week of low shear stress pre-conditioning enhanced the retention and viability of seeded ECs(191).

The laboratory of Matsuda has done considerable work with synthetic scaffolds: using both Dacron and polyurethane. When an artificial ECM of collagen with dermatan sulphate was pressurized through the Dacron scaffold and then lined with ECs, 100% patency at up to 16 weeks, was achieved in canine carotid arteries, though adding SMCs to the ECM improved EC retention and ECM production(192;193). Using a similar model but including fibroblasts with SMCs in the ECM resulted in over 80% patency in canine carotids at periods of up to 23 weeks(194-196). Using segmented polyurethane as the scaffold onto which the ECM of collagen and dermatan sulphate was squeezed through, followed by EC seeding showed 75% patency up to 26 weeks when implanted into canine carotids(197-199). As with Dacron, the ECM layer could have had SMCs added(200). More recently endothelial progenitor cells (EPCs) derived from canine peripheral blood were pre-lined onto a collagen mesh which was then wrapped with a segmented polyurethane film. Out of 12 grafts implanted into canine carotids, 11 remained patent at up to 3 months(201). Ratcliffe also used

polyurethane scaffolds seeded with SMCs and then ECs which were cultured under fluid flow and finally implanted into the carotid arteries of dogs with 100% patency to four weeks(174).

Sparks(202) lined PTFE with peritoneum, making sure the visceral surface with mesothelium was luminal. When implanted into rabbit carotid artery the 21-day patency was 80% compared to 20% for the contralateral carotid, which had PTFE without the mesothelial lining.

2.4.2 Moulds and Mandrels

As mentioned earlier moulds and mandrels simply confer a mechanical framework so that the physical proportions of the ensuing graft resemble that of the vessel they are imitating.

Matsuda and colleagues developed a tubular hybrid medial tissue by pouring a cold mixed solution of SMCs and type I collagen into a corresponding tubular mould and by subsequent thermal gelation, followed by seven days of culturing and finally seeding with ECs(203;204). Unfortunately, burst pressures were relatively low at up to 100mmHg so that even when used as a venous conduit an outer Dacron reinforcement was used: over a 24 week period 9 of 14 canine posterior vena cavas remained patent (205). More compliant outer reinforcements such as segmented polyester and polyurethane-nylon meshes showed that polyester had better compliance and burst pressure was maximal when kept on the outside of the collagen-SMC layer rather than inside or within it(206). Furthermore this method could be used to generate branched bypasses(207). More recent work from this group using microporous segmented polyurethane as the external reinforcement, especially with a high pore density, showed patency rates of 100% over a six month period in canine carotid arteries(208;209).

Berglund used a variety of cross-linking techniques on type 1 collagen to produce an acellular sleeve around which a second layer of collagen with neonatal dermal fibroblasts was moulded. Mechanical properties like burst pressure and tensile stress were significantly enhanced – though not to native arterial levels - by every crosslinking treatment, especially glutaraldehyde crosslinking. However, glutaraldehyde limited cell ingrowth into the acellular layer and had a negative morphological impact on the endothelial cells seeded on the inner acellular crosslinked sleeve. Furthermore these typically brittle glutaraldehyde-crosslinked sleeves ruptured. The authors therefore hypothesized that physical rather than chemical crosslinking of collagen with ultraviolet radiation and dehydrothermal treatment may offer better results(210).

L'Heureux produced an innovative graft exclusively from cultured human cells. Firstly an acellular lining made by dehydrating a fibroblast sheet was wrapped around a PTFE mandrel and then another sheet, this time made of cultured SMCs was wrapped around this. After maturing in a bioreactor an outer sheet of fibroblasts was added before further maturation. Then the inner PTFE mandrel was removed and seeded with ECs. These grafts were inserted into dogs though without ECs, which were felt to be too antigenic in a xenotransplant model. Despite this precaution patency was only 50% at one week(211). Campbell, working on rats and rabbits developed a vascular graft by inducing an inflammatory reaction. The method involved inserting a silastic tube into the peritoneal cavity, which then acted essentially as a mandrel around which layers of myofibroblasts, collagen matrix and a single layer of mesothelium developed over a two week period. The tube of living tissue was then extracted and everted after the silastic tube was removed and the graft was then inserted into the aorta of the same animal. The patency rate was 67% over a

four month period(212). Tsukagoshi working with rabbits employed a similar method. A piece of fascia from the thigh was excised and wrapped around a silicone rod and then implanted into a subcutaneous pocket in the thigh. After four weeks the fascia-wrapped silicone rod was excised and the rod was removed. The fibrocollagen tube was then treated with protamine, glutaraldehyde and heparin and then anastomosed onto the femoral artery with patency rates of over 70% without any aneurysms(213).

2.5 Improving Scaffold-Cell Adhesion

One of the key elements to any tissue-engineered blood vessel is retaining the cells on the scaffold itself. The initial failure of single-stage seeding of endothelial cells to improve patency of prosthetic vessels was felt to be because upon exposure to pulsatile blood flow a high proportion of cells are washed off(111), especially in the first 30-45 minutes where up to 70% cells are lost, this is followed by a slower exponential loss over the next 24 hours and then normally a levelling off period(114). Some PTFE grafts have shown EC attachment of only 10±7% with only 4±3% of the ECs retained(116). Therefore much work has been expended on improving cell adherence to scaffolds for both ECs and now also SMCs. This includes both physical and chemical methods.

2.5.1 Large Molecules

Historically, the main chemical methods for improving cell-scaffold adhesion involved the use of coatings or pre-clot. A detailed review of the various options for improving EC attachment to scaffolds has been done by Salacinski et al(111). The main coatings that have been used are collagen, fibronectin (FN), laminin, poly-l-lysin and gelatin. Of these FN would appear to be the best(214;215), though better results are obtained if it is used in combination with collagen, fibrin, laminin, gelatin or extracellular matrix(216-218). Recently the immobilization of biomacromolecules like gelatin and collagen has been enhanced by introducing free amino groups (NH_2) onto polyurethane membranes with consequent enhancement of EC proliferation(219).

For SMC adhesion, the main work has been on attachment to plastic culture dishes rather than scaffolds. Here fibronectin, collagen and to a lesser extent laminin and

vitronectin have improved adhesion(220). SMC adhesion and spreading on a potential biodegradable scaffold [made of poly(propylene fumarate-co-ethylebe glycol) and agmatine-modified poly(ethylene glycol)-tethered fumarate hydrogels] was enhanced after protein modification increased the amount of the positively–charged guanidine group of agmatine(221;222).

EC adhesion and retention has been enhanced even more by pre-clotting prosthetic grafts(111) using the patient's own plasma(116;223) or blood(136;224;225). Pre-clotting with serum was less successful(116;224). However, the best method seems to be a combination of fibrin glue and fibroblast growth factor(226).

Chemical bonding of surface moieties such as peptides has shown some promising results. Heparin was the first to be tried and has shown mixed results in improving EC adhesion(227;228). Lectins have shown excellent results(229) but perhaps the most intriguing possibilities lie with RGD peptides.

2.5.2 Peptides

RGD is a tripeptide sequence (Arginine-Glycine-Aspartate) found in extracellular matrix proteins like fibronectin. It is the binding motif for cell surface integrin receptors and has been investigated extensively(111;230-233). Our laboratory has shown that RGD when covalently bonded to CPU and particularly when this was in association with heparin, significantly enhances the retention and viability of seeded ECs(141), others have also produced enhanced cell retention onto grafts using RGD peptides(234).

RGDs have also been used to enhance SMC attachment to scaffolds. RGDS (Arg-Gly-Asp-Ser) when incorporated into a hydrophilic gel matrix successfully entrapped SMCs throughout this artificial vascular media(235). RGD peptides when grafted onto dextran as a biomaterial surface coating demonstrated increased SMC attachment

and spreading(236). KQAGDV, which is derived from the γ chain of fibrinogen, appears to be a mimic of the RGD sequence because its binding to integrins is inhibited by RGD peptides, though it binds primarily to the α rather than β subunit of integrins.(237). KQAGDV significantly enhance SMC attachment to modified surfaces and hydrogel polymer scaffolds, though this is at the expense of reduced cell proliferation(233) and ECM production(230).

2.6 Summary

The above review demonstrates the varied approaches that have been undertaken to produce a TEBV (see also Table 2.2). All have their advantages and disadvantages. 'Off-the-shelf' vessels have their major attraction in being suitable for emergency and urgent cases. The possible options include the animal-derived natural scaffolds, but concerns about immunity and transmission of animal diseases especially prions currently restrict their attractiveness.

A fully autologous blood vessel made from human cells which is both mechanically strong and chemically responsive has only been demonstrated by L'Heureux (211;238). Short-term animal studies proved difficult because of problems of immunity and consequently there are no long-term studies. Synthetic scaffold systems offer the reassurance of a strong structure resistant to aneurysm formation – critical for vessels exposed to high arterial pressures over long periods of time. However, that invariably limits their compliance and increases their infection risk. Perhaps the most promising area is that of biodegradable scaffolds, which have even been taken to a clinical level in the low-pressure pulmonary circulation(239-241), though long-term results are still awaited. All current approaches have two major limitations though, in that they all require a separate operation to access or extract the cells needed followed

by a long period of culturing or developing these cells. Again improvements to the scaffold-cell interaction with the use of peptides may shorten the culture period as well as ensuring greater cell retention.

Therefore the ideal TEBV has yet to be found and it may well be that the 'ideal' will vary depending on the clinical situation. Assuming future experiments are successful and repeatable, fully autologous vessels or vessels with a biodegradable scaffold could be offered when there is plenty of time before an operation is required. For more urgent cases 'off-the-shelf' grafts based on synthetic or animal scaffolds may prove superior to traditional Dacron or PTFE.

In order to avoid the risks of aneurysm formation with both fully autologous TEBV and biodegradable scaffolds, the work in this thesis used a permanent scaffold. In order to avoid the risk of compliance mismatch the scaffold used was compliant and matched the mechanical properties of vessels in the peripheral circulation.

Furthermore to overcome risks of graft thrombogenicity, a layer of endothelial cells was used to line the lumen. This endothelial cell layer was supported by a layer of smooth muscle cells

SCAFFOLD / MANDREL	ECM	Cells	Peptides	Patency	Researcher
Porcine SIS & Col.		None	Heparin	100%≤90/7	HUYUNH(178)
Porcine / Dog SIS		None	None	≥75%≤5 yrs	LANTZ(177)
Decel. Porcine Aorta		MyoFB & EC ≈ $2x10^6$/cm length	None	Not assessed	BADER(179)
Decel. Porcine Iliac Vessel		EPC: confluent layer	None	100%≤130/7	KAUSHAL(182)
Decell. Bovine Ureter		None	None	100%≤10/12	CLARKE(180)
Decell. Porcine Carotid		None	Heparin	100%≤67/7	CONKLIN(181)
PGA/PDX	None	None	None	≥86%%≤12/12	GREISLER(186)
PGA	None	SMC: $5x10^6$/ml EC: $3x10^6$/ml	None	100%≤24/7	NIKLASON(188)
PGA-PHA	None	≈ $10^6/cm^2$ mixed SMC, EC & FB	None	100%≤150/7	SHUM-TIM(189)
PU	None	≈ $2x10^6/cm^2$ SMC	None	92%≤1/52	YUE(187)
PTFE	Peritoneum		None	80%≤21/7	SPARKS(202)
Dacron	Col.	0.2-30x10^5SMC/ml & 10^5EC/cm^2	+/- FN	N/A	WEINBERG(190) BAGUNEID(191)
PU/ Dacron	Col. + Dermatan sulphate	6.6x10^5EC/cm^2 +/- 7.5-20x10^5/ml SMC +/- 7.5x10^5/ml FB	None	75-100% 16-26/52	MATSUDA ET AL(193;194;199)
PU	Col.	EPC	None	92%≤ 3/12	HE(201)
PU	None	SMC & EC	None	100%≤4/52	RATCLIFFE(174)
ST	ECM + MyoFB & Mesothelial Cells secondary to inflammatory reaction		None	67%≤4/12	CAMPBELL(212)
Fascia-wrapped ST			Protamine& Heparin	73%≤8/52	TSUKAGOSHI(213)
PTFE	None	SMC & FB sheets +/- EC	None	50%≤1/52	L'HEUREUX(211)
Glass	Col.	FB: 10^6/ml & EC: $25x10^3/cm^2$	None	N/A	BERGLUND(210)
Glass + Mesh	Col.	5-15x10^5/ml SMC & 4x10^5EC/cm^2	None	64-100% ≤6/12	MATSUDA ET AL(205;207;208)

Table 2.2: Previous Tissue-Engineered Bypass Conduits

Key: Col.=Collagen; Decel.=Decellularised; EC=Endothelial Cells; FB=Fibroblasts; PGA=polyglycolic acid; PDX=polydioxanone; PHA=polyhydroxyalkanoate; ST=silicone tube; PU=Polyurethane; SIS=Small Intestinal Submucosa; SMC=Smooth Muscle Cells

Times: x/7 = x days; y/52 = y weeks; z/12 = z months; yrs = years

Chapter 3

EXTRACTION AND ASSESSMENT OF VASCULAR SMOOTH MUSCLE CELLS FOR TISSUE ENGINEERING

3.1 Introduction

The SMC is the critical cell of the middle layer of the artery, being principally responsible for the artery's ability to respond to various stimuli causing it to either dilate or constrict. This in turn determines distal blood flow and also affects the blood pressure of the body as a whole. Therefore, researchers have focussed on extracting and modulating SMCs for development of their Tissue-Engineered Blood Vessel (TEBV).

This chapter will initially review the literature on SMC extraction and assessment of their functionality. In doing this I have focussed on human cell sources to maintain my theme of developing a clinically-applicable hybrid graft. Thereafter the chapter will investigate and characterise the SMCs extracted from human long saphenous vein and umbilical cord using a novel technique developed in-house at BTEC.

3.1.1 Sources of Human Vascular Smooth Muscle Cells

Sources of hVSMCs (human vascular smooth muscle cells) can be subdivided into arterial and venous. Either of these can be sourced from cadavers, superfluous tissue from operative specimens or tissues no longer needed by the body (e.g. – umbilical cord specimens).

In developing methodologies applicable to hybrid graft development, the source of hVSMCs must be a blood vessel which is not critical to life or body function. The only arterial specimens which fit this criteria are the radial, gastroepiploic and inferior epigastric arteries(31). However, the operative extraction of the gastroepiploic artery represents a significant risk to health itself as it requires an abdominal laparotomy and so should not be considered as a cell source. The inferior epigastric artery is variable

in size and can be technically difficult to dissect out(31). This leaves the radial artery, but this can only be used if there is sufficient collateral circulation from the ulnar artery, as determined by Allen's Test(31).

Venous sources are numerous as there are many superficial veins of significant size to be extracted without significant morbidity. The principal examples include the long and short saphenous veins, cephalic, brachial and external jugular veins.

For my purposes ethical and consent issues excluded the use of cadaveric vessels. An alternative was getting vessels from bypass procedures which regularly use the saphenous veins and occasionally the radial artery, but the amount of 'spare' vessel is insufficient to use this as a cell source for research purposes.

This leaves tissues no longer needed by the body. The two main examples of this are the long saphenous vein removed during varicose vein surgery and umbilical cord vessels. Both these vessels were used as cell sources.

3.1.2 Extraction Methods for hVSMCs

There are two main methods(242-244) to extract hVSMCs from blood vessels: explantation(245) and enzymatic digestion(246).

A) Explantation

The vessel is initially cleaned by removing fat and connective tissue and then cut longitudinally. The endothelial layer is then removed usually mechanically with a sterile cotton wool bud. Then explants are either dissected out from the media or punched out with a biopsy punch before being transferred to culture dishes or flasks. These are then placed inside an incubator, with minimal amounts of culture medium to prevent the explants from floating off the surface. Over the next 48 hours sufficient

extra medium is added to prevent dessication of the explants. Thereafter a full volume of medium is added to the explants.

B) Enzymatic Digestion

The vessel is again cleaned of fat and connective tissue. The adventitial and endothelial layers are then removed either mechanically or enzymatically using collagenase. This leaves the vessel media which is then exposed to a combination of digestive enzymes consisting of collagenase, elastase, hyaluronidase and trypsin. There are a variety of enzyme concentrations and time periods to which the vessels are exposed. After this the cell solution is centrifuged and the cell pellet resuspended in culture medium before plating out onto a culture dish or flask(242;244;246;247).

3.1.3 Nature of SMCs

SMCs exist in two forms: synthetic and contractile(242-244;248). In the normal resting state, within a blood vessel, they are in the contractile state functioning to maintain vascular tone and responding to both mechanical and chemical stimuli. In this state, contractile filaments occupy 75-80% of the cell cytoplasm, with few organelles like rough endoplasmic reticulum, Golgi apparatus and free ribosomes. In contrast the synthetic state is seen in development, repair, myointimal thickenings and atherosclerotic lesions. In this state the SMC functions to replicate and produce extracellular matrix (ECM) and is characterised by few myofilaments but large amounts of rough endoplasmic reticulum, Golgi apparatus and free ribosomes(244). SMCs freshly extracted from their blood vessel start in a contractile state(244). They can then be stimulated into the synthetic state by growth factors in the culture medium: usually found within the serum, though a whole host of factors can stimulate this change(249).

Over the first 1-2 days, SMCs resembling fibroblasts attach onto the substrate surface, taking on a spindle-shape. They then extend cytoplasmic processes laterally and flatten down. This is when the SMC undergoes considerable reorganisation of its interior towards a synthetic phenotype. After 5 days, the SMCs begin their logarithmic growth phase(250). This phenotypic shift is inhibited if the cells are seeded at high density. In contrast, at very low seeding densities, there is evidence that this shift is irreversible(251). It is worth remembering however to be cautious about extrapolating findings from one blood vessel in one animal to a different blood vessel in another animal species(242). It must also be stated that there is a continuum of cell types between contractile and synthetic: for example some cell types whilst still contractile can divide(252).

SMCs when grown to confluency in culture appear under phase contrast light microscopy in a hill-and-valley conformation(244). Immunohistochemical analysis reveals the presence of α-actin, myosin and tropomyosin(244). Typically, contractile SMCs have low levels of α-actin and high levels of myosin, a situation reversed in synthetic SMCs(249). In contrast endothelial cells are positive for von Willebrand Factor, factor VIII antigen, thromboplastin, CD31 and CD34.

To assess the feasibility of lining a graft with a layer of SMCs, it is critical to know how long it takes to generate sufficient cells from the time of extraction to graft seeding. This requires an assessment of the time it takes for SMCs to change from a contractile to a synthetic state – this is derived from the time taken to achieve confluency of the first culture flask. Thereafter growth can be assessed over time through different passages until sufficient cells are produced. From this information, a population doubling time can be calculated.

3.1.4 Ability of SMCs to Contract Collagen

The ability of SMCs to return to a contractile phenotype is important as these cells are responsible for a vessel's vasoreactivity. One way of doing this *in vitro* is to see if extracted SMCs are able to contract collagen in two-dimensional flat collagen gels, even after a rapid proliferative phase. Furthermore, Vitamin C (Ascorbic Acid) is known to stimulate ECM production by SMCs(253) and so enhance the strength of the resulting matrix(211).

3.2 Materials and Methods

3.2.1 Extraction of SMCs

At BTEC, we have developed a methodology for combining the approaches(254;255) of explantation and enzymatic digestion as outlined below. For saphenous vein samples, ethical approval had been gained from the Ethics Committee of the Royal Free Hospital and informed consent was sought and gained from all participating patients.

The method requires a set of surgical instruments consisting of a scalpel, scissors and two pairs of non-toothed forceps which are sterilised by autoclave beforehand. Then the following protocol is carried out.

Protocol (Appendix 1)

1) Collect long saphenous vein or umbilical vein / artery specimen into sterile phosphate buffered saline (PBS) + 0.2% gentamicin (Sigma G-1397) solution.
2) Trim ends of vessel.
3) Cut to 15cm.
4) Strip adventitia manually by grasping one end of the vein and pulling the adventitia away either by everting or degloving it from the remaining vein.

5) Flush lumen with PBS + gentamicin solution.
6) Place in centrifuge tube with 7.5ml of sterile Dulbecco's modified eagle medium (DMEM) (Sigma D6171) with 0.1% collagenase A (Boehringer Mannheim 103 586) + 0.05% elastase (Sigma E-6883). Enzyme solutions are sterilized first through 0.2 micron filters (Nalgene 190-2520).
7) Agitate for 45 minutes at 37°C in an orbital mixer (Denley OM301).
8) Transfer vein into 7.5ml of fresh enzyme solution.
9) Agitate for 2 hours at 37°C.
10) Transfer vein into universal container with 20ml DMEM + 0.04% collagenase.
11) Agitate overnight for 16 hours at 37°C.
12) Remove remaining vein and place onto 25cc flask as an explant.
13) Carefully transfer solution to centrifuge leaving behind any visible vein particles.
14) Centrifuge at 140G for 7 minutes (MST Mistral 1000).
15) Pipette off supernatant.
16) Resuspend the cell plug in 1ml Smooth Muscle Cell (SMC) medium*.
17) Remove 40 µl for cell counting.
18) Add further 6ml SMC medium.
19) Transfer to 25cm^2 culture flask and incubate at 37°C in 5% CO_2 atmosphere.

* SMCs were serially cultured in SMC tissue culture medium. This medium consisted of DMEM [Dulbecco's Modified Eagle's Medium] with HEPES modification, 10% FBS [Foetal Bovine Serum], 2mM L-Glutamine, 2.5 µg/ml Amphotericin B, 100 units/ml penicillin and 100 µg/ml streptomycin.

3.2.2 Characterisation of SMCs

Confirmation that the extracted cells were indeed SMCs included studying the appearance of cells under light microscopy and immunohistochemistry.

Digital photographs of growing SMCs were taken at confluency through a phase contrast microscope to assess their growth pattern.

Immunohistochemical analysis of the cells was performed to confirm the presence of key cell-specific antigens. Cells were therefore assessed for the following immunohistochemical markers:

1. SMC: alpha-actin.
2. EC: von Willebrand Factor.
3. Fibroblasts.
4. Positive Control: vimentin.
5. Negative Control: normal mouse serum.

Protocol

The cells were identified with the marker antibodies to the antigens outlined above. The ABC Vectastain kit was used according the following protocol:

1) Cytospins were made of the cells.
2) The cytospins were fixed with acetone and then stored in a freezer until the day of staining.
3) On the day of staining the slides were defrosted for 30 minutes at room temperature.
4) A ring around the cells was formed with a paraffin pen.
5) Horse serum was applied on the cells at a concentration of 1/100 for each slide for 20 minutes and then washed with PBS.

6) vWF was used at dilution of 1/50, fibroblast antibody was used at a dilution of 1/100 and actin, normal mouse serum and Vimentin were used at a concentration of 1/500. The primary antibody was used for 1 hour and then washed with PBS.
7) 2nd reagent was then prepared as follows: 1μm of normal horse serum and 1μm of universal biotinylated in 50μm of PBS per slide.
8) At the same time the tertiary reagent was also prepared using 1μm of reagent A and 1μm of reagent B in 50μm of PBS.
9) 2nd reagent applied for 30 minutes and then washed with PBS. Tertiary reagent was applied for 30 minutes and then washed with PBS.
10) Using the Vector Peroxidase Kit, 5ml of water + 2 drops of buffer and 4 drops of DAB and 2 drops of hydrogen peroxide were mixed. This was applied on the slides for 5-7 minutes.
11) The slides were immersed in H&E for 4 minutes, washed, dipped in alcohol and then removed immediately. They were immersed in bluing solution for 2 minutes, washed and then mounted with coverslips.
12) Again digital photographs were taken of the resultant stains, which were converted into photomicrographs using Adobe Photoshop.

3.2.3 Growth of SMCs

The growth characteristics of the two SMC sources used - umbilical cord and saphenous vein – was investigated over numerous passages to establish:

1. Time to initial confluency – how long it takes for the SMCs from initial extraction to become confluent for the first time.

2. Population doubling time – how long it takes for the average SMC to duplicate. This was derived from the cell counts taken at confluency and the time it took to reach confluency.
3. Saturation density – the density of cells when confluency in the flask is achieved.

3.2.4 Ability of SMCs to Contract Collagen

To determine the ability of SMCs to contract collagen gels, the following protocol was followed. To optimise the contraction of collagen the effect of varying concentrations of SMCs and Type 1 Collagen was assessed. Furthermore the effect of Vitamin C (Ascorbic Acid) on both speed of collagen contraction and weight of resultant collagen discs was studied in a separate experiment by adding 50μg/ml of Vitamin C to half the solutions of SMC Medium.

Protocol

1. The collagen solution was prepared in the ratio below:

Collagen (in acetic acid)	: 7.5% bicarbonate	: 10 x DMEM
6 parts	: 3 parts	: 1 part

2. Prepare SMC into various cell concentrations:

   E.g. from 2 x 10^4 SMC/ml to 1 x 10^6 SMC/ml.

3. Fill wells of a 6-well plate with the above concentration of cells in SMC medium and collagen solution in ratio of 2:1.
4. Incubate for 1 hour at 37°C humidified 5% CO_2.
5. Free collagen gel from edges of plate with sterile needle.
6. Return to incubator.
7. Measure contraction every 24 hours.

8. Change solution with fresh SMC medium every 48 hours.

Data Analysis and Statistical Methods

Data are presented with mean ± standard deviation (SD). For the cell growth data comparing the SV & UC groups; and the collagen weight data comparing groups with and without Vitamin C the two-tailed T-test was used.

3.3 Results

3.3.1 Extraction of SMCs

The protocol outlined in section 3.2.1 succeeded in 60% of saphenous vein (6 out of 10) and 50% of umbilical cord (6 out of 10) extractions, the failures were usually due to technical errors.

Although cell counts were attempted, the number of contaminating cells made cell counts unreliably high and so this was abandoned in later attempts. Indeed for the explant part of the experiment, cell counts can't be done at all.

3.3.2 Characterisation of SMCs

Figure 3.1 is a digital photograph through a phase contrast microscope showing the typical hill-and-valley appearance of confluent SMCs in culture.

Figure 3.2 is a digital photograph of immunohistochemical stains on the extracted SMCs which demonstrates that the cells stained positive with α-actin (SMC stain), but negative for both von Willebrand Factor (EC stain) and Fibroblast marker to confirm their SMC status.

Figure 3.3 shows these images in more detail using photomicrographs of the digital images with the positive and negative controls for comparison.

The immunohistochemical analysis was done for four different SMC populations.

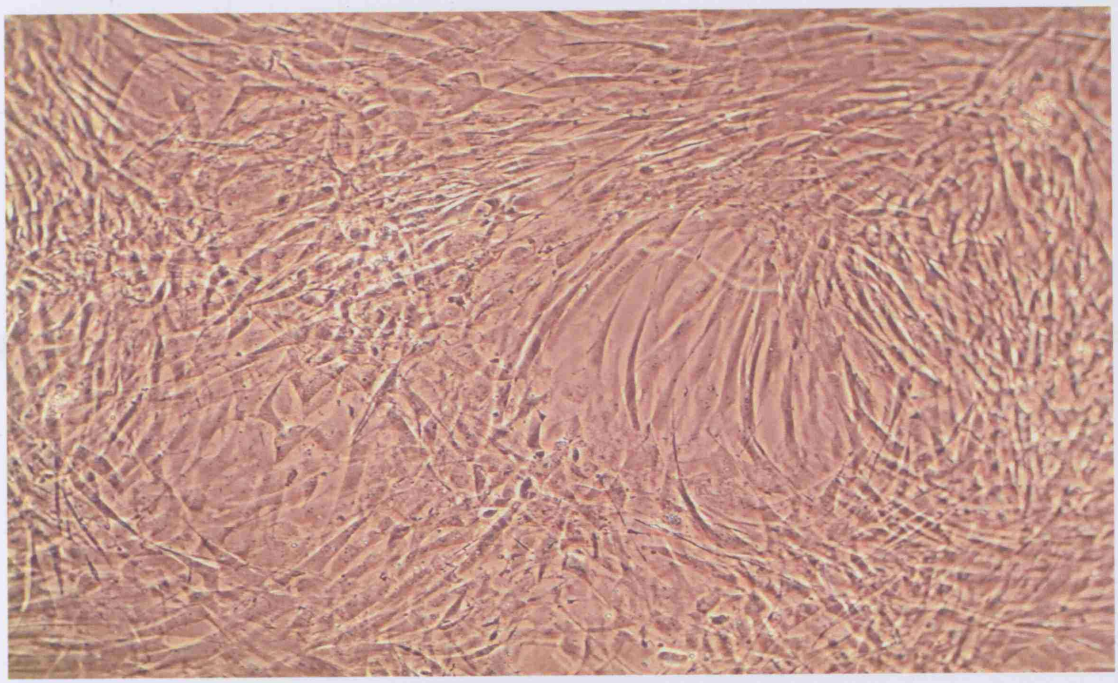

Figure 3.1: Smooth Muscle Cell Growing in Classical Hill and Valley Formation

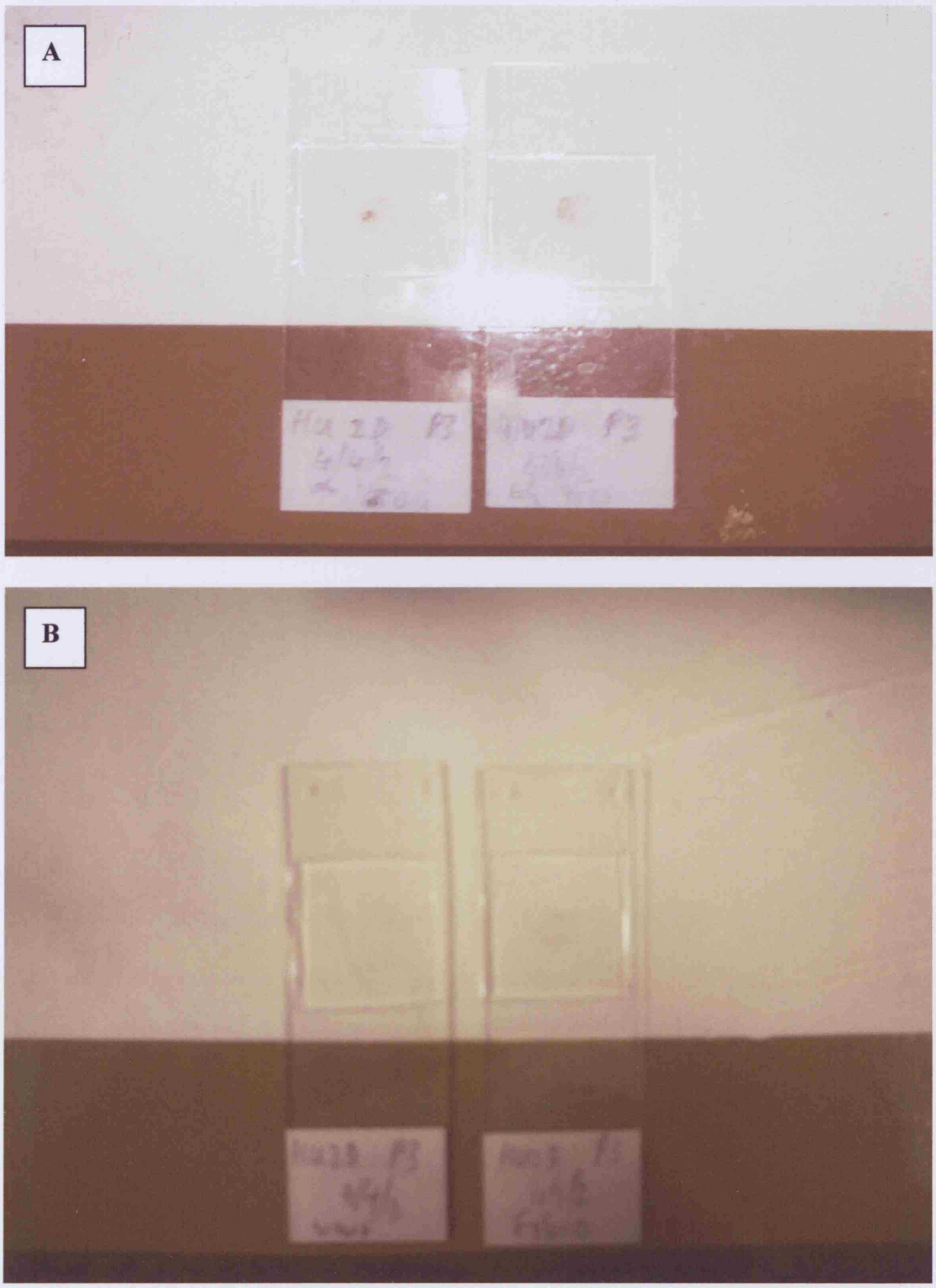

Figure 3.2: Smooth Muscle Cell Staining

(A) Alpha-actin staining: positive - staining clearly visible on slides

(B) Von Willebrand Factor & Fibroblast staining: negative - no stain visible on slides

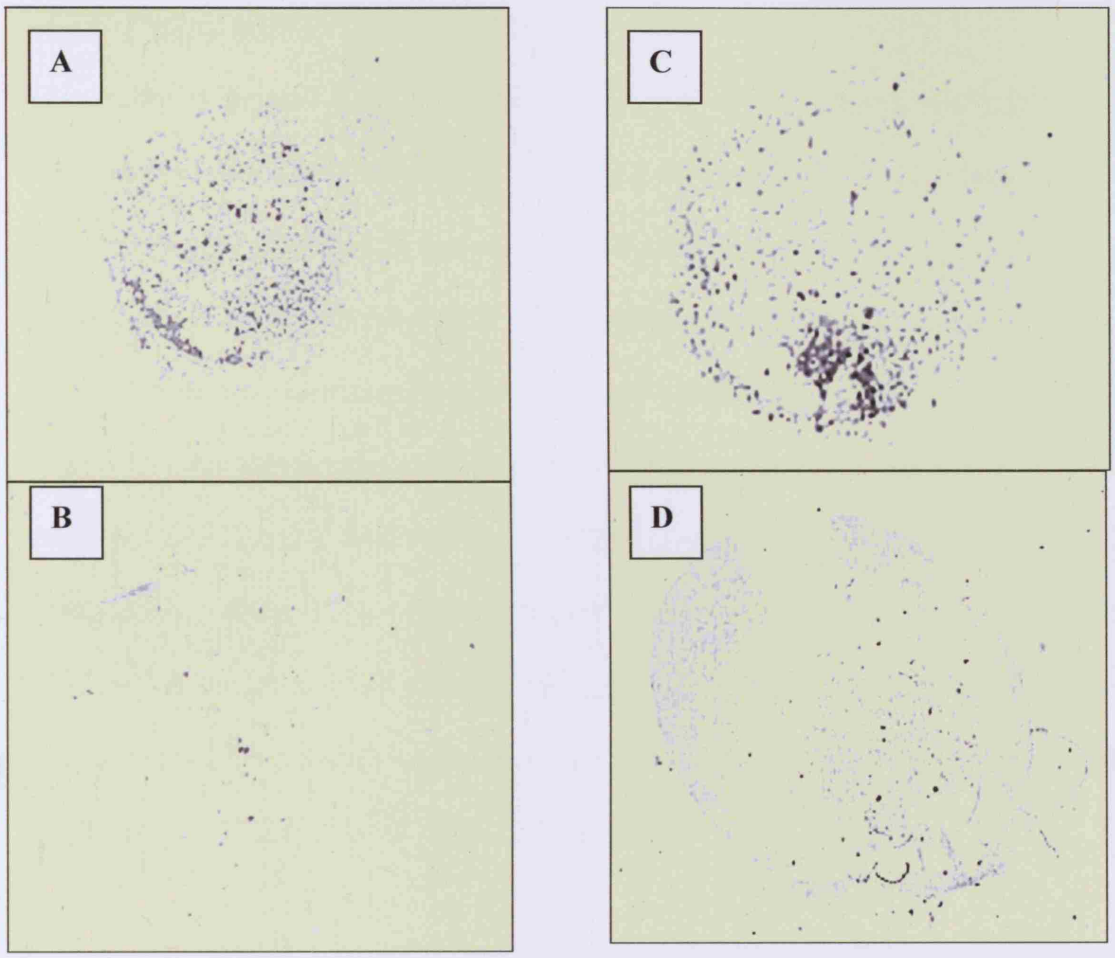

Figure 3.3: Photomicrographs of Smooth Muscle Cell Staining

(A) Vimentin: Positive Control

(B) Normal Mouse Serum: Negative Control

(C) Alpha-actin: Positive

(D) Von Willebrand Factor: Negative

Photomicrographs of images from Figure 3.2. Image 3.3C stains positive confirming the smooth muscle cell epitope whereas image 3.3D shows a lack of staining for an endothelial cell epitope.

3.3.3 Growth of SMCs

As is shown in Figure 3.4 (a) & (b), the SMCs follow a logarithmic growth curve. The time for initial confluency was quicker (13.3±4.2 days against 25.2±16.1 days; n=6: p=0.0330 [T-test] - Figure 3.5) and population doubling time shorter (3.4±0.6 days against 5.6±1.9 days; n=8: p = 0.0227 [T-test] - Figure 3.6) for SMCs extracted from umbilical cord. However, there was no difference between the two sources of SMCs in terms of saturation density as determined by SMC concentration per cm^2 at confluency ($3.7\pm2.3x10^4$ SMC/cm^2 for umbilical cord against $3.6\pm1.6x10^4$ SMC/cm^2 for saphenous vein; n=17: p = 0.8105 [T-test] - Figure 3.7).

In Figure 3.4, the starting cell numbers for saphenous vein varied from $1.6 x10^5$ to $4.2x10^5$ cells - Figure 3.4(a), whereas for umbilical cord extractions the numbers varied from $7 x10^4$ to $6.75x10^6$ cells – Figure 3.4(b).

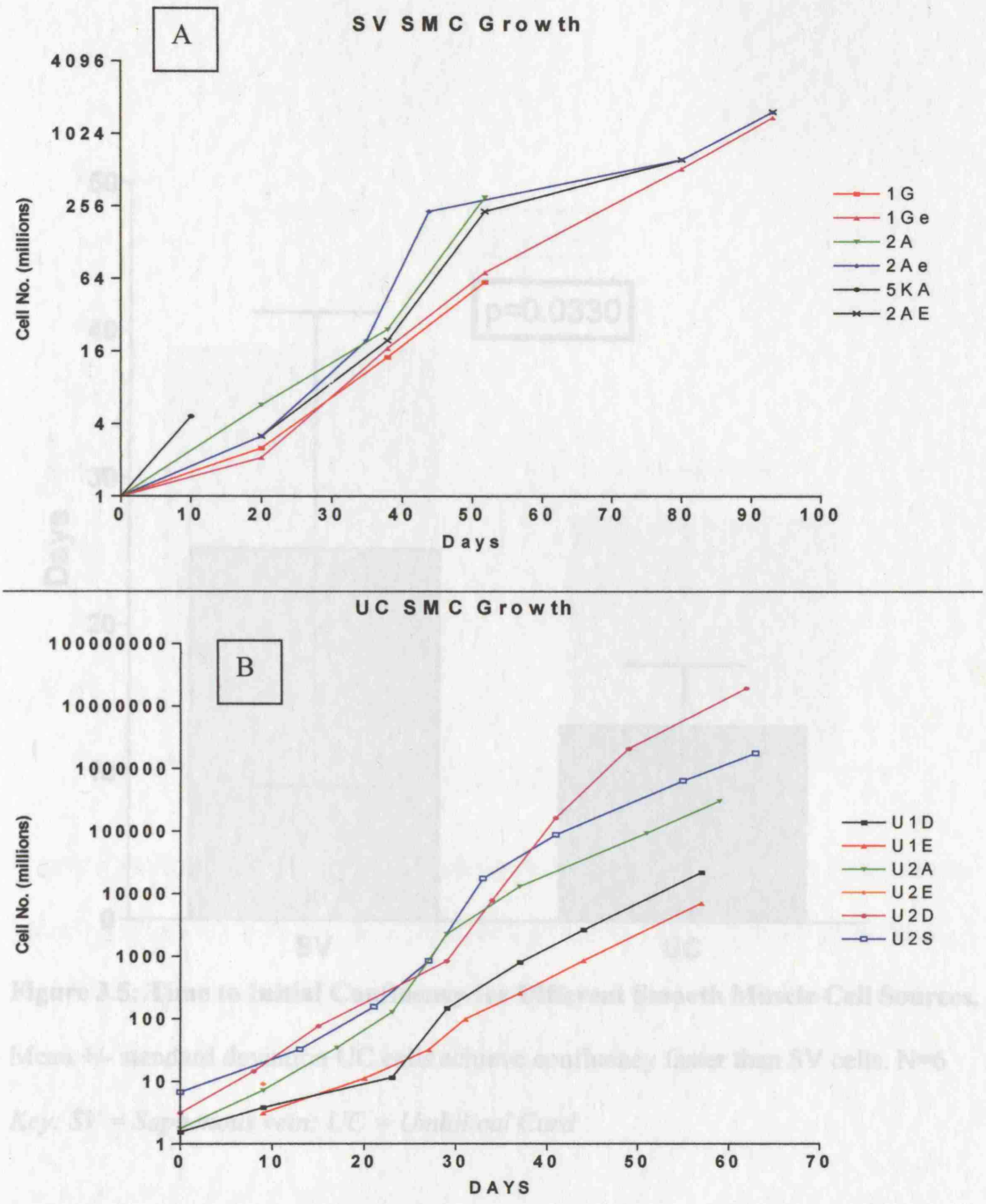

Figure 3.4: Growth Curves for Different Smooth Muscle Cell Sources

(A) Saphenous Vein (SV); (B) Umbilical Cord Vessels (UC)

Key: 1G – 2AE & U1D – U2S = SMC Populations from Different Patients

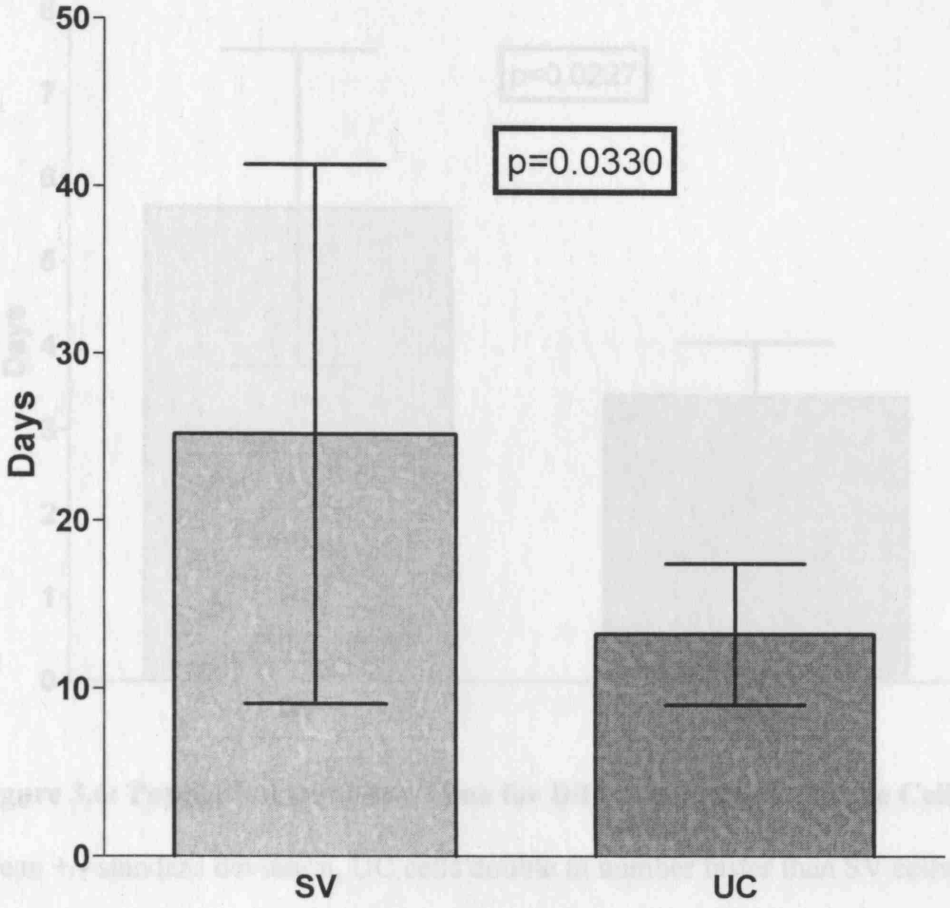

Figure 3.5: Time to Initial Confluency for Different Smooth Muscle Cell Sources.

Mean +/- standard deviation UC cells achieve confluency faster than SV cells. N=6

Key: SV = Saphenous vein; UC = Umbilical Cord

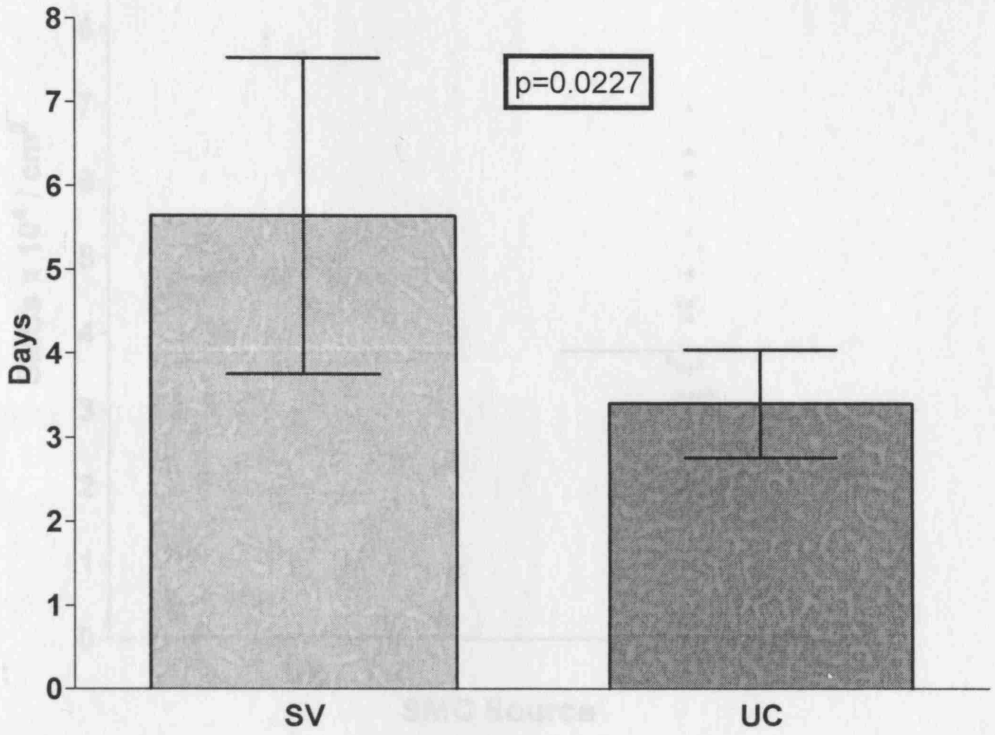

Figure 3.6: Population Doubling Time for Different Smooth Muscle Cell Sources

Mean +/- standard deviation. UC cells double in number faster than SV cells. N=8

Key: SV = Saphenous vein; UC = Umbilical Cord

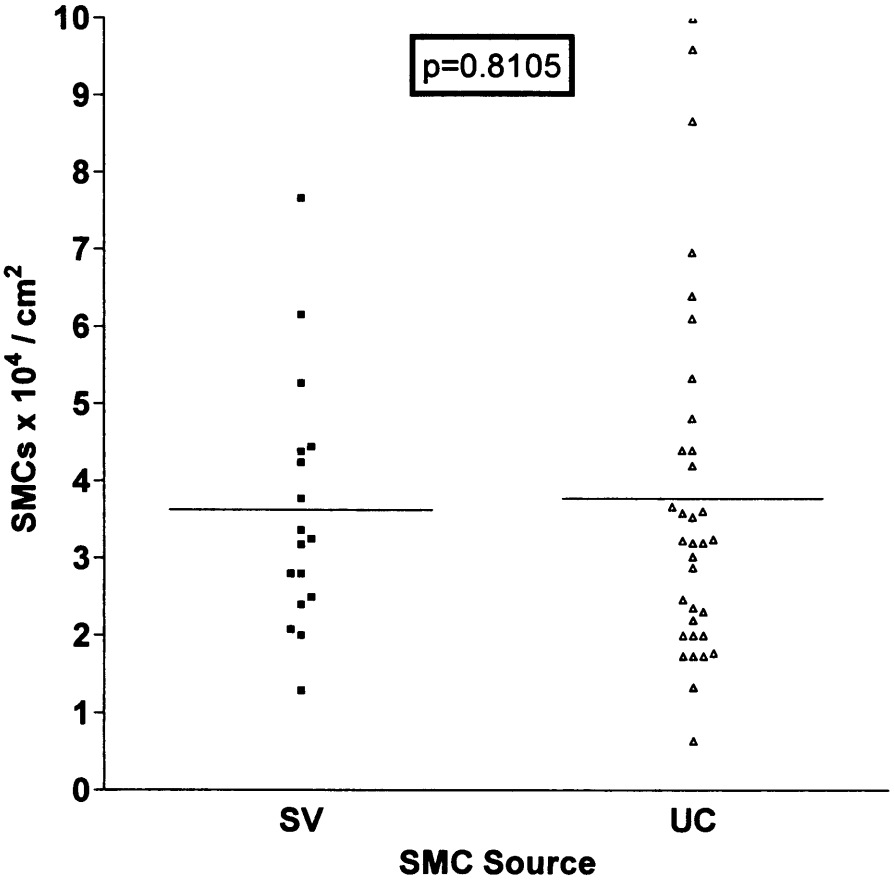

Figure 3.7: Saturation Density for Different Smooth Muscle Cell Sources

Mean and scattergram. No significant difference was found between UC and SV cells. N=17. *Key: SV = saphenous vein; UC = umbilical cord.*

3.3.4 Ability of SMCs to Contract Collagen

Extracted SMCs irrespective of passage number were able to successfully contract collagen gels. Photographs of the contracting collagen gel are shown at day 1 in Figure 3.8(a) and day 7 in Figure 3.8(b)

As can be seen from the data shown in Figure 3.9 collagen contraction is slower with low SMC concentration ($2x10^4$ SMC/ml). The graphs for $1x10^5$ and $1x10^6$ SMC/ml are almost identical. However, by 7 days there is little difference between any of the SMC concentrations.

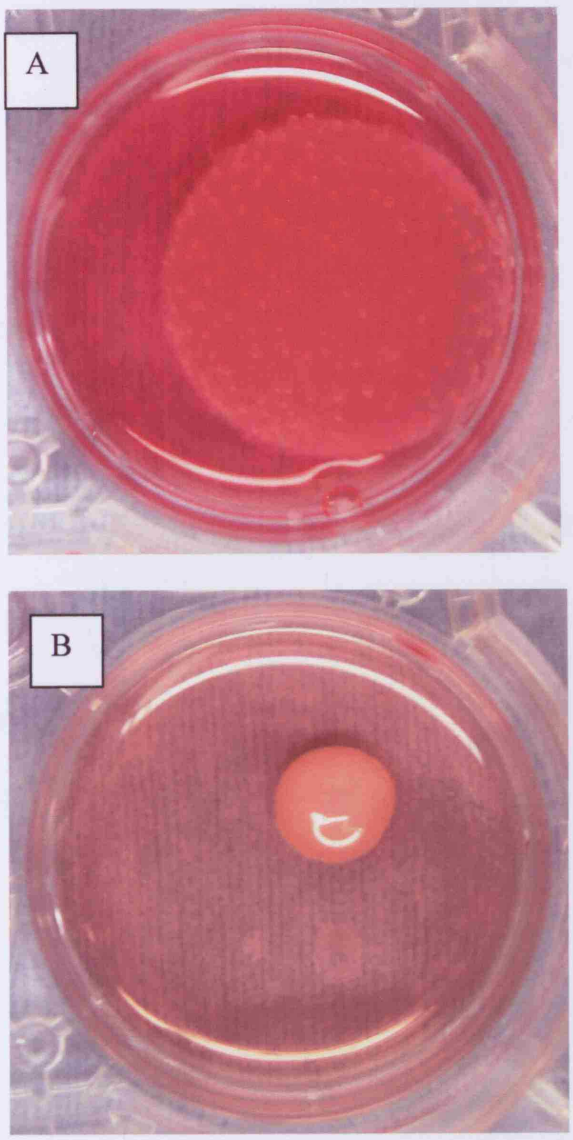

Figure 3.8: Contracting Collagen Gels

Key: A = after 1 day of contraction; B =after 7 days of contraction.

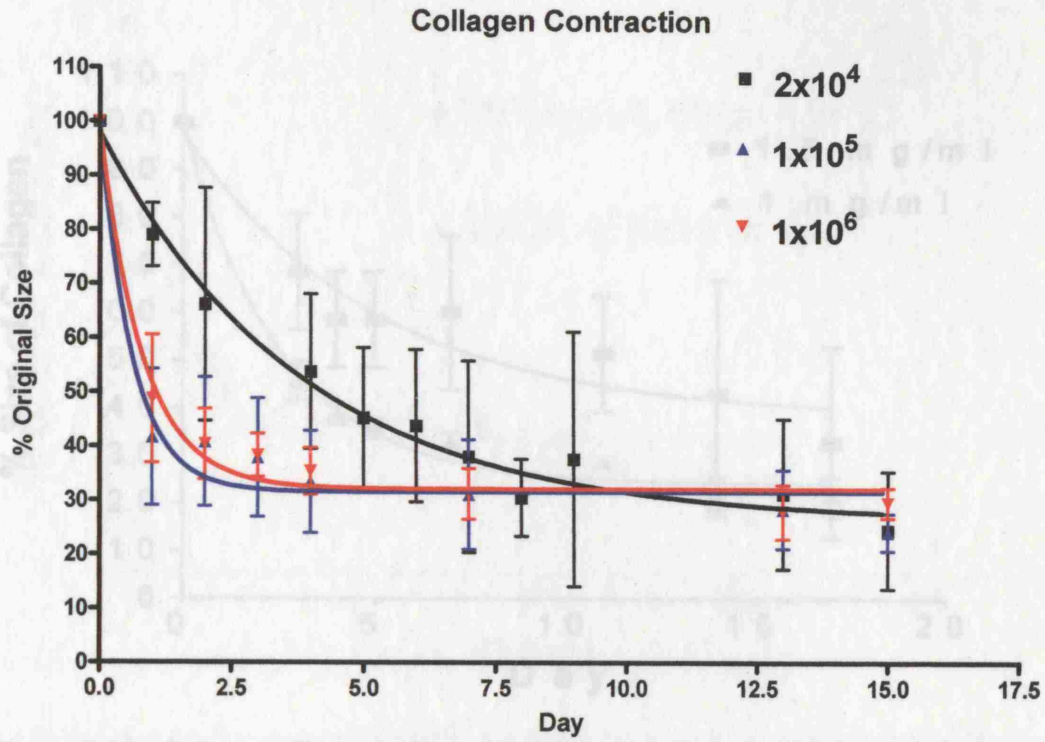

Figure 3.9: Collagen Contraction at Varying Smooth Muscle Cell Concentration

Higher cell concentrations result in faster collagen contraction.

Key: Mean +/- standard deviation. Concentrations in SMC per ml

The concentration of collagen also affected the speed of contraction, although after 2 weeks the differences became non-significant (Figure 3.10).

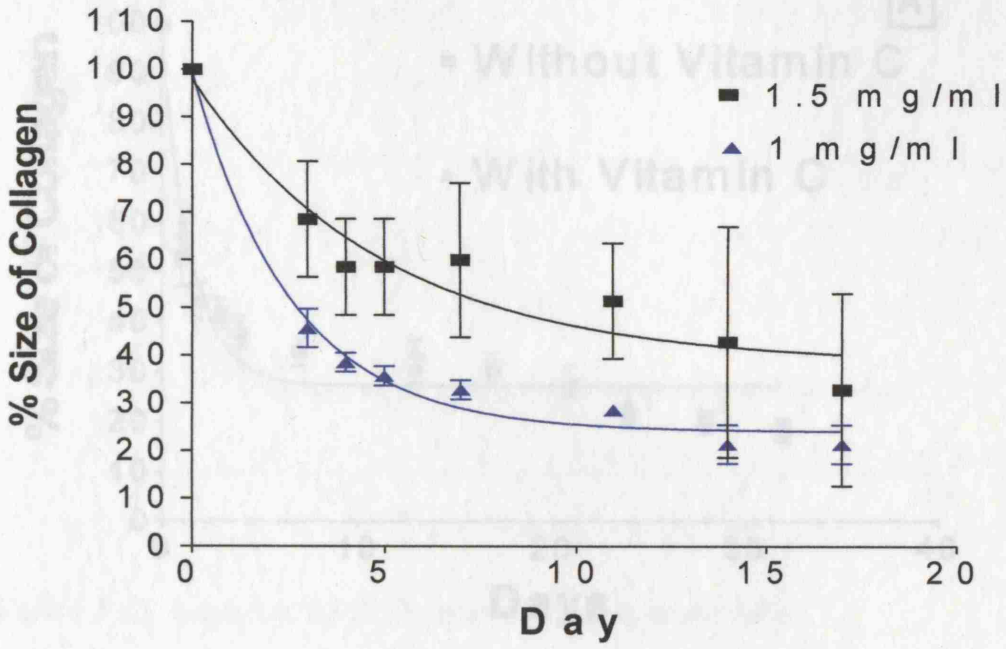

Figure 3.10: Collagen Contraction with Different Collagen Concentrations.

Lower collagen concentration had faster contraction rate.

Key: Mean +/- standard deviation

Vitamin C had no impact on collagen contraction rate and did not seem to increase extracellular matrix production as determined by wet (p=0.3102; T-test) and dry (p=0.9785; T-test) weights of the collagen discs - Figure 3.11. There was no difference in speed of contraction between SMCs whether from UC or SV as shown in Figure 3.12 below.

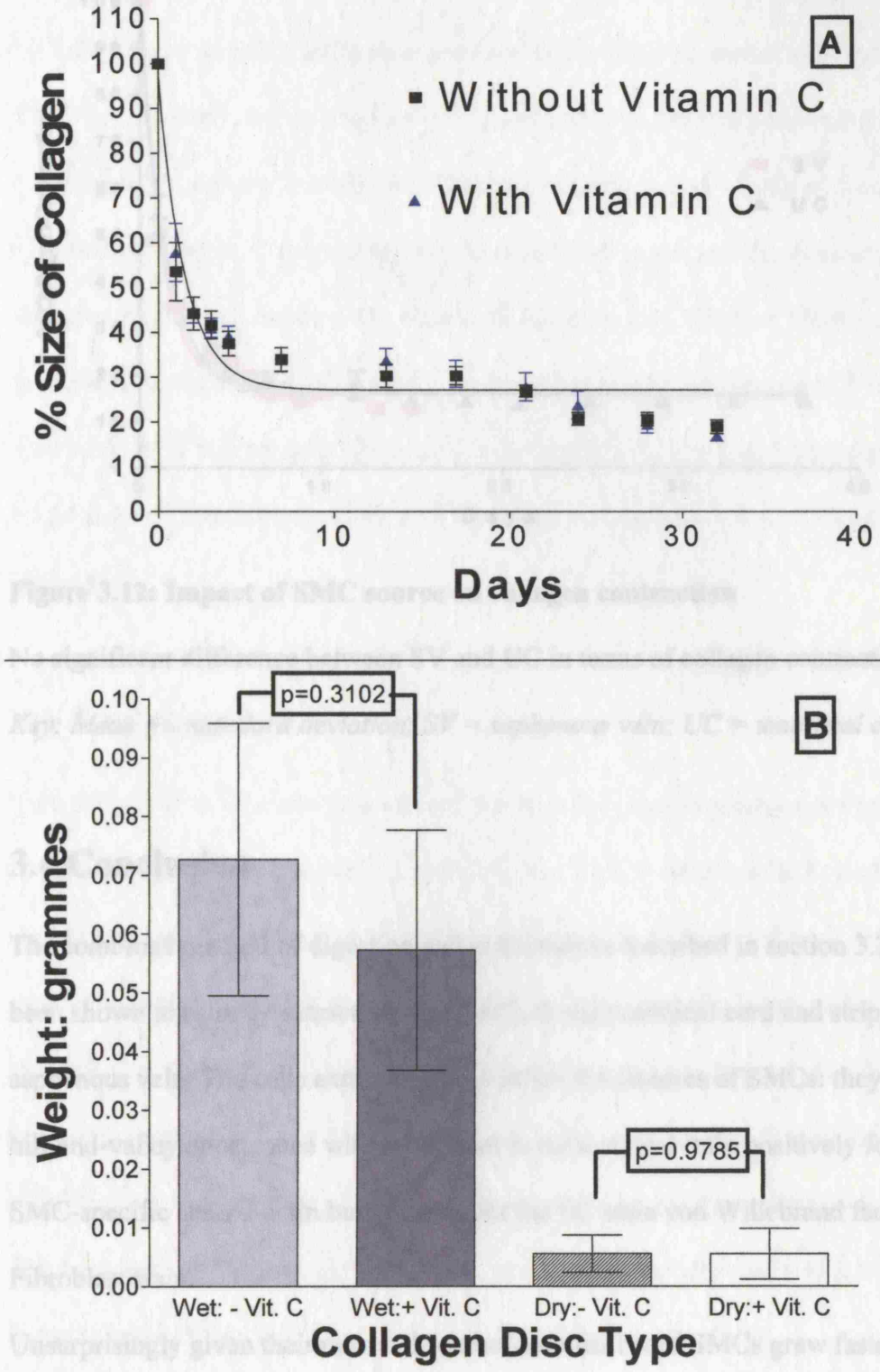

Figure 3.11: Impact of Vitamin C on Collagen Contraction

(A): Contraction Curve over Time. (B): Collagen Disc Weights

No impact of Vitamin C on either collagen contraction or disc weight.

Key: Mean +/- standard deviation.

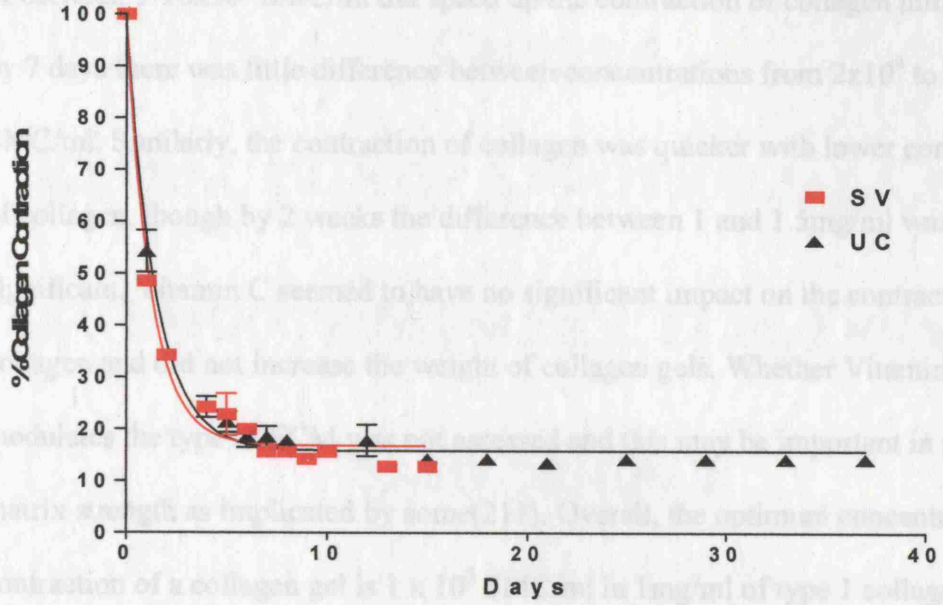

Figure 3.12: Impact of SMC source on collagen contraction

No significant difference between SV and UC in terms of collagen contraction.

Key: Mean +/- standard deviation; SV = saphenous vein; UC = umbilical cord

3.4 Conclusion

The combined method of digestion and explantation described in section 3.2.1 has been shown to reliably extract SMCs from both the umbilical cord and stripped saphenous vein. The cells extracted demonstrate the features of SMCs: they show the hill-and-valley appearance when confluent in culture; and stain positively for the SMC-specific stain α-actin but negative for the EC stain von Willebrand factor and Fibroblast stain.

Unsurprisingly given their neonatal source, umbilical cord SMCs grew faster than adult saphenous vein SMCs.

SMCs extracted from human saphenous vein and umbilical cord can transform into a synthetic phenotype, be passaged several times and yet retain their ability to return to a contractile phenotype and contract down collagen. Higher concentrations of SMCs

of between 1-10x10^5 SMC/ml did speed up the contraction of collagen initially, but by 7 days there was little difference between concentrations from 2x10^4 to 5x10^5 SMC/ml. Similarly, the contraction of collagen was quicker with lower concentrations of collagen, though by 2 weeks the difference between 1 and 1.5mg/ml was not significant. Vitamin C seemed to have no significant impact on the contraction of collagen and did not increase the weight of collagen gels. Whether Vitamin C modulates the type of ECM was not assessed and this may be important in terms of matrix strength as implicated by some(211). Overall, the optimum concentration for contraction of a collagen gel is 1 x 10^5 SMC/ml in 1mg/ml of type 1 collagen without Vitamin C.

3.5 Summary

This chapter describes how one can extract vascular smooth muscle cells from human tissues that are readily available. Umbilical cord SMCs achieve initial confluence sooner and grow faster than saphenous vein SMCs – a reflection of their origin from a growing neonate. Both are capable of returning to the contractile state after serial passaging as demonstrated by successful contraction of collagen gels and this is optimally achieved using a SMC solution of 1x10^5 SMC/ml and 1mg/ml of collagen. Finally, no obvious benefit of adding Vitamin C to the growth medium was found. The next step in the building of a hybrid graft requires the discovery of a reliable human source of both SMCs and ECs ideally from the same tissue.

Chapter 4

EXTRACTION OF ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS FROM THE SAME SOURCE

4.1 Introduction

Endothelial cells (EC) are the critical cell of the luminal surface of blood vessels. As mentioned in chapter 1 ECs by themselves have been used to successfully improve the patency of prosthetic grafts(119;120) because of their anticoagulant properties and inhibition of excessive SMC proliferation. Therefore any hybrid graft should contain the key cells of the middle layer (SMCs) and the luminal surface (ECs). An obvious source would therefore actually be a blood vessel itself.

This chapter will describe the extraction of human ECs from blood vessels and the feasibility of a sequential method for extracting ECs and SMCs from the same vessel.

Endothelial cells have successfully been extracted from human veins and arteries. The veins used in humans are primarily the saphenous and external jugular veins though forearm veins and arteries have also been used(91). The saphenous vein is only used if it is unsuitable as a graft itself - less than 3mm in diameter, stenosed or atheromatous.

The techniques used in cell extraction are cannulation, eversion, scraping and mincing.

4.1.1 Cannulation

In cannulation, one end of the blood vessel is clamped, the other end is cannulated, an enzyme solution is added through the cannula and the vessel is then clamped and incubated at 37°C(98). Incubation times vary between 10-30 minutes. Prolonged incubation leads to loosening of non-endothelial cells such as smooth muscle cells. At the end of the incubation period, the enzyme and cell mixture is pored out then

neutralised with serum and centrifuged. The EC pellet is then re-suspended ready for use or further culturing.

4.1.2 Eversion

The eversion method involves everting the vessel over a sterile rod, the enzyme solution being added onto the everted surface and incubated as per the cannulation method(256). The mixture is then collected and centrifuged as per the cannulation technique.

4.1.3 Scraping

Here steel wool is used to scrape the lumen of the vessel to remove ECs. This technique was used in the first reported single-stage seeding procedure in the 1970's(112). Alternatively a commercially-available cell scraper can be used.

4.1.4 Mincing

One technique utilised less often involves chopping the vessel into fine fragments before enzymatic/non-enzymatic degradation(257;258).

Whilst mechanical techniques like mincing and scraping were amongst the first to be used, their relatively poor cell yields have resulted in a preference for the enzymatic cannulation and eversion methods(84;259). Bourke et al compared the cannulation technique with the eversion technique in extracting ECs from the jugular vein of sheep and demonstrated a significantly higher yield of cells with the cannulation technique(260).

Cannulation is therefore the preferred technique in human vessels. With cannulation, greater than 95% cell viability can be achieved in human trials(90) and venous EC yields range from $0.06-0.91 \times 10^5$ cells/cm^2(84). It is worth noting though that ECs fail to proliferate in culture in 5-27% of cases and even when culture succeeds the mean

number of days of culture required to achieve enough cells to coat a 70cm long PTFE graft (6 mm internal diameter) is 25±11 days(261). Furthermore many patients requiring bypass surgery are older, uraemic from renal failure and smokers - factors known to significantly reduce the number of cells extracted and prolong culture time before the proliferation phase(85).

For the purpose of the following experiments the restriction as stated in chapter 3 to vessels no longer needed by the body still applied: long saphenous vein (SV) removed during varicose vein surgery and umbilical cord (UC) vessels. Both these vessels were used as cell sources.

4.2 Methods and Materials

Initially the cannulation method was used for extracting cells from both the SV and UC. The eversion and scraping techniques were also used for SV. Mincing was felt to be inappropriate as the technique needed to be able to extract ECs and SMCs separately from the same vessel and mincing would have made this an impossible task.

A sample of stripped SV was also sent for SEM (scanning electron microscopy).

4.2.1 Cannulation Protocol

1. Collect long saphenous vein or umbilical cord specimen in sterile phosphate buffered saline (PBS) + 0.2% gentamicin (Sigma G-1397) solution.
2. Trim ends of vessel.
3. Cannulate both ends of the vessel and tie cannulas.
4. Flush vessel lumen with PBS until no more blood in washings.
5. Confirm vessel wall is intact and if holes are found either trim vessel further beyond hole or repair with 5/0 prolene.

6. Fill lumen with sterile M199 (Gibco) containing 0.05% collagenase A (Boehringer Mannheim 103 586).
7. Clamp off both ends and gently massage vessel.
8. Incubate for 10 minutes at 37°C.
9. Massage vessel again to release luminal ECs.
10. Release clamps and pour luminal contents into centrifuge tube.
11. Wash through lumen with Complete Medium twice again collecting contents in centrifuge tube.
12. Centrifuge luminal contents at 300g for 5 minutes.
13. Re-suspend cell pellet and pour into 25cc tissue culture flask with 6ml of Complete Medium and place in 37°C / 5% CO_2 incubator.

4.2.2 Eversion Protocol

1. Collect long saphenous vein specimen in sterile phosphate buffered saline (PBS) + 0.2% gentamicin (Sigma G-1397) solution.
2. Trim ends of vessel.
3. Flush vessel lumen with PBS until no more blood in washings.
4. Strip adventitia.
5. Evert whole length of vessel so lumen is now exposed on the outside.
6. Place vessel in 50cc sterile container half-filled with sterile M199 (Gibco) containing 0.05% collagenase A (Boehringer Mannheim 103 586).
7. Agitate container with vessel.
8. Incubate for 10 minutes at 37°C.
9. Gently massage vessel lumen to release luminal ECs.
10. Pour contents of container into centrifuge tube.

11. Submerge vessel with Complete Medium twice again collecting contents in centrifuge tube.
12. Centrifuge collected washings at 300g for 5 minutes.
13. Re-suspend cell pellet and pour into 25cc tissue culture flask with 6ml of Complete Medium and place in 37°C / 5% CO_2 incubator.

4.2.3 Scraping Protocol

1. Collect long saphenous vein specimen in sterile phosphate buffered saline (PBS) + 0.2% gentamicin (Sigma G-1397) solution.
2. Trim ends of vessel.
3. Flush vessel lumen with PBS until no more blood in washings.
4. Strip adventitia.
5. Cut longitudinally along whole length of vessel so lumen is now exposed.
6. Use sterile cell scraper (Marathon Laboratories) to remove ECs along whole of vessel lumen.
7. Place scrapings into centrifuge tube filled with Complete Medium.
8. Centrifuge collected scrapings at 300g for 5 minutes.
9. Re-suspend cell pellet and pour into 25cc tissue culture flask with 6ml of Complete Medium and place in 37°C / 5% CO_2 incubator.

4.3 Results

4.3.1 Cannulation Protocol

For Umbilical Cord this proved a very successful method with a success rate of 67% (8 out of 12).No attempt was made to count the number of cells yielded as many of the cells are not EC and it proved impossible to distinguish different cell types when

looking down a microscope onto a haemocytometer. Confirmation of the unreliability of initial cell counting after extraction was demonstrated when cell numbers at extraction exceeded those at first passage despite a period of culturing.

Unfortunately for stripped saphenous vein this was a complete failure with none of six vessels yielding ECs. The major problem was the holes left by avulsed side branches of SV from the stripping of the vessel. Even when holes were repaired with 5/0 prolene to prevent leakage of the enzyme solution out of the lumen, after ten minutes in the incubator there would always be some leakage out of the lumen. Therefore the luminal surface was not fully expanded to allow full exposure of the endothelial surface to digestive enzymes. This contrasted with the UCs where one could see the lumen under expansile pressure of the enzyme solution even after ten minutes in the incubator.

4.3.2 Eversion Protocol

This method despite overcoming the problem of needing a completely intact vessel wall did not yield any ECs in 6 attempts.

4.3.3 Scraping Protocol

This method despite overcoming the problem of uncertainty of contact between the EC-containing luminal surface and a digestive enzyme solution also did not yield any ECs in 2 attempts.

After failing to extract any ECs from the SV samples despite trying different methodologies, a sample of stripped SV when sent for scanning electron microscopy revealed an absence of recognisable ECs on the luminal surface (Figure 4.1).

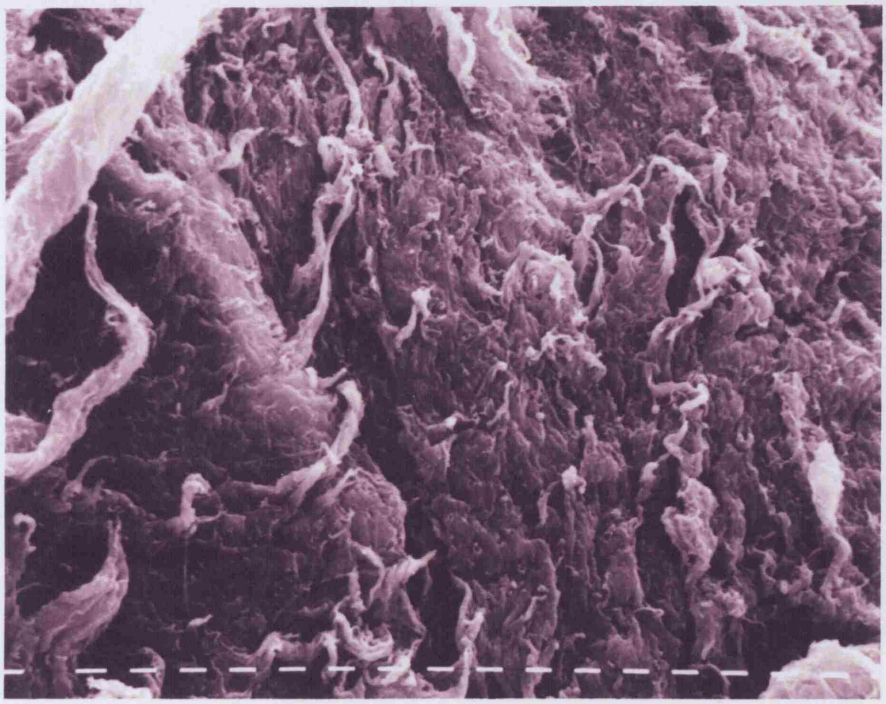

Figure 4.1: Scanning Electron Micrograph of Stripped Varicose Vein Lumen

Note the absence of endothelial cells on the vessel lumen confirming the severe effect of stripping on the endothelial cell layer

4.4 Conclusion

The Cannulation method of filling the lumen of a vessel with digestive enzymes successfully extracts ECs from UC. Unfortunately neither of the attempted methods – cannulation, eversion and scraping – yielded an EC population that could be cultured from stripped SV. The SEM results (Figure 4.1) would suggest that this is because SV when stripped is denuded of most if not all ECs. This contrasts with the success of using non-stripped SV which does yield ECs. The surgical method followed does not involve the use of a stripper down the vessel lumen, but the careful dissection out of the whole vessel, thus saving the luminal surface from any trauma. Specimens include excess vein harvested for arterial bypass grafting principally for coronary artery surgery(98;262) but also for peripheral bypass(90;92).

In order to source ECs from SV, given the failure of stripped samples, one would need to get unused lengths of SV from patients undergoing bypass surgery. However, the need for a 15cm length to extract SMCs also means that this would be unacceptable ethically. An alternative surgical technique for varicose vein patients uses a procedure that effectively avulses the SV along its length. This method again preserves the EC luminal lining but requires multiple cuts along the length of the vessel and is not a familiar method for most surgeons - so again posing an ethical dilemma that was felt to be unacceptable.

The results of these experiments meant that henceforth only UC could be used for dual cell – EC and SMC – extraction.

4.5 Summary

The results of the experiments in this chapter have demonstrated that vascular endothelial cells and smooth muscle cells can be extracted sequentially from human umbilical cord. Unfortunately none of the other techniques employed – cannulation, eversion or scraping - could extract endothelial cells from stripped saphenous vein. The explanation for this lies in the fact that the surgical technique for saphenous vein extraction probably denudes the inner lining of the vessel of most of its endothelial-cell layer. Therefore this could not be used as a source for experiments to develop a hybrid graft, as a well-preserved vein would be required for harvesting of ECs. Further development of the hybrid graft requires the investigation of how best to attach cells to the compliant scaffold.

Chapter 5

ROLE OF CELL ATTACHMENT PEPTIDES, CELL CONCENTRATION AND ATTACHMENT PERIOD IN OPTIMISING SMOOTH MUSCLE CELL ADHERENCE TO COMPLIANT POLY(CARBONATE-UREA)URETHANE SCAFFOLDS

5.1 Introduction

This chapter describes the development of a hybrid graft using compliant poly(carbonate-urea)urethane (CPU) as a simple scaffold onto which SMCs and extra-cellular matrix (ECM) develop.

The chemistry and manufacture of the CPU graft have already been published(263). In brief, it is made of poly(carbonate-urea)urethane extruded via low temperature cast coagulation. As mentioned in Section 1.3.2(A), the CPU graft has a honeycomb structure that allows it to maintain compliance similar to that of human artery(136) and pulsatile flow *in vivo* through a mechanism of wall compression that accommodates increases in volume without the need for external dilation. This compares to previous polyurethane based grafts that exhibited a compliance mismatch caused by perivascular ingrowth(140). The CPU graft has been shown to have a superior ability to attach ECs, which can be further improved by pre-lining the graft with attachment factors like collagen and fibronectin(118;136;139;140). The graft has undergone *in vitro* degradation tests and has been implanted in a dog model for 36 months, demonstrating very high biostability(133-135). This graft is already in use as an A-V fistula for haemodialysis access and is undergoing a phase I clinical trial as a peripheral vascular bypass graft(133). Furthermore studies have shown that EC attachment can be enhanced by pre-lining synthetic grafts with SMC(187).

Essentially all peptides promoting cell adhesion are derived from sequences found in ECM molecules. Of these fibronectin (FN) and vitronectin are the primary serum glycoproteins promoting cell adhesion, with FN being the most studied(264). Fibronectin is a glycoprotein ($\sim$ 250,000 Da) with a modular structure. It contains domains for binding fibrin, heparin, gelatin, collagen, EC, and has cysteine and

arginine-glycine-aspartate (RGD) residues. Although it significantly improves EC retention, fibronectin is susceptible to hydrolysis(264). Critically the amino acid sequence Arg-Gly-Asp (RGD)(265) in the tenth type III repeat of FN serves as a primary cell attachment cue(266). RGD is the most studied and effective peptide for cell adhesion on synthetic surfaces, based upon its widespread distribution within organisms, its ability to address more than one cell adhesion receptor and its biological impact upon cell anchoring, behaviour and survival. Furthermore cell adhesive RGD sites have been found in other ECM proteins like vitronectin, collagen, fibrinogen, von Willebrand factor, laminin, membrane proteins and even viral and bacterial proteins(267).

Vascular SMCs (vSMCs) contain a host of cell adhesion receptors including integrins, syndecans, alpha-dystroglycan, cell adhesion molecules and cadherins(268).

Cadherins are cell-cell adhesion receptors whose role in vSMCs is uncertain(268).

Cell adhesion molecules belong to the immunoglobulin family of cell-cell adhesion receptors with a transmembrane protein. In vSMCs two adhesion molecules, intercellular adhesion molecule (ICAM-1) and vascular cellular adhesion molecule (VCAM-1), have been detected in vascular disease states(269), although ICAM-1 can bind fibrinogen and hence participate in ECM-cell interactions(268).

Syndecans are members of a proteoglycan family of transmembrane adhesion receptors with important roles in cell-ECM and cell-cell adhesion and migration(270;271). Alpha-dystrophin is a proteoglycan receptor for the dystrophin-glycoprotein complex, which provides mechanical strength for the cell membrane during muscle cell contraction, linking the external basement membrane with the internal actin filaments.(268).

Integrins are the main cell receptors for ECM proteins like FN, laminin, collagens and vitronectin, and so are the main mediators of cell-cell and cell-ECM adhesion(266). Integrin-ECM interactions are highly promiscuous: there is evidence that upon binding different integrins, the same ECM protein (e.g. – FN) can mediate different functions and one integrin can bind multiple ligands. Integrins are heterodimers of two non-covalently associated glycoproteins, α and β: there are 18α and 8β subunits combining to form at least 24 heterodimers(272). About half have been shown to bind to ECM molecules in an RGD-dependent manner(267).

Integrin-mediated cell adhesion comprises a cascade of four overlapping events, beginning with cell attachment which occurs within seconds to minutes. This allows cells to withstand gentle shear forces, whereas without integrin-mediated attachment cells could easily be rinsed off a surface. The next event is for the cell to flatten as its cell membrane spreads to take its characteristic shape. Thirdly, actin is organised into microfilament bundles also known as stress fibres. Finally focal adhesions or contacts are formed, consisting of clustered integrins and other transmembrane, membrane-associated and cytosolic molecules – these link ECM molecules to the cell's actin cytoskeleton and are important in mediating transmembrane signalling(266). It is well established that integrin-mediated cell spreading and focal adhesion formation trigger survival and proliferation of anchorage dependent cells(273). Loss of attachment causes apoptosis in many cell types (called "anoikis")(267).

The aims of this study were to determine if attachment factors could enhance the adhesion of SMCs to the CPU, the concentration of cells that give optimum attachment to CPU and the impact of time on attachment of cells to CPU.

5.1.1 Role of Cell Attachment Peptides

A host of cell attachment peptides were selected, especially those based on the RGD peptide and variations of fibronectin, to try to improve SMC attachment to CPU. Arginine-glycine-aspartate (RGD) is the tripeptide adhesion receptor for matrix proteins such as fibronectin or laminin, which enhance cell attachment. RGD has demonstrated improved cell attachment in ePTFE grafts compared to fibronectin, whilst also having antithrombogenic effects, which would make it potentially superior to fibronectin(231;232;274;275)

Fibronectin adhesion promoting peptide (FAPP), fibronectin-like engineered protein polymer (FEPP) and fibronectin-like engineered protein polymer plus (FEPP+) may enhance cell attachment and retention. FAPP is a sequence found in the carboxy-terminal heparin-binding domain of fibronectin and is known to possess a small charge. FEPP is a polymer that incorporates thirteen identical copies of the ten amino acid, cell-attachment epitope (containing RGD) from human fibronectin interspersed between structural peptide sequences engineered to have a high positive charge(276), this positive charge is increased further in FEPP+. Positively charged surfaces are more conducive to cell adhesion and morphological maturation(277;278). Therefore using highly charged adhesive agents might show promise.

5.1.2 Role of Cell Concentration & Attachment Time

The impact of various concentrations of cells on attachment and the optimal concentration needed to cover the surface needs to be determined.

Cell concentrations of $2x10^5$ SMCs per cm^2 were used as this has been the lowest recommended concentration to use on CPU graft using endothelial cells(118;141;279). At higher concentrations, the surface area available for each cell

to attach may have been insufficient, making any benefit from different attachment factors harder to detect.

In addition the impact of time on the attachment of cells is important in developing a confluent layer which remains attached to the CPU surface. Therefore, the CPU was lined with varying concentrations of SMCs for varying time periods. Because the CPU graft does not allow light to readily pass through it, cell coverage of the graft can not be reliably determined with light microscopy, so images of radiolabelled cells using a phosphoimager were taken.

5.2 Methods and Materials

Human umbilical vein and arterial smooth muscle cells (SMC) were extracted using a novel enzymatic extraction process developed in-house(254) as outlined in Appendix 1.

Cell Radiolabelling Protocol (Appendix 2)

Cells were radiolabelled according to the following protocol that we have developed(280) with 1.8 MBq ^{111}In-oxine (Amersham International, Amersham, Bucks UK) per 10^6 cells.

1. Count cells to be radiolabelled.
2. Re-centrifuge to form cell pellet and pour off supernatant.
3. Re-suspend cells in 4ml of serum-free medium: Medium 199 or DMEM.
4. Add ^{111}Indium-Oxine at 1.8MBq/10^6 cells and incubate at 37°C for 15 minutes.
5. Re-centrifuge cell suspension at 300G for 7 minutes.

6. Pour off supernatant and re-suspend cells in 4 mls of serum-containing medium.
7. Incubate at 37°C for 5 minutes and repeat stages 5-7 twice.
8. Finally cells were ready to be diluted to an appropriate concentration, ready for seeding.

The cells were used when confluent on the sixth passage after trypsinization and resuspension in SMC medium to obtain the cell count. The cells were eventually diluted in SMC medium to the concentration necessary to achieve a seeding density of $2.25x10^5$ cells/cm^2.

5.2.1 Role of Cell Attachment Peptides

Lining CPU with Attachment Factors and Radiolabelled SMCs

All CPU grafts were 4mm in internal diameter. Lengths of CPU were filled with the following biomacromolecules:

1. RGD (ARG-GLY-ASP) at 633μg/ml.
2. Superfibronectin at 42μg/ml.
3. Fibronectin at 118μg/ml.
4. Fibronectin-Like Engineered Polymer Protein at 118μg/ml.
5. Fibronectin-Like Engineered Polymer Protein Plus 133μg/ml.
6. Type 1 collagen at 1mg/ml (all from Sigma-Aldrich).
7. A control batch consisted of native graft without a coating.

The grafts were then left for 24 hours.

The next day the grafts were cut along their length and then into 1.5 cm long pieces. These CPU segments were then placed in 2ml syringes with the plungers holding

them in position and the CPU lumen facing upwards. The syringes were filled with 0.7ml of SMC solution at $2x10^5$ cells/ml.

After 48 hours in an incubator, the solution was aspirated off and the CPU inner lumens were lavaged with sterile phosphate buffered saline (PBS). The aspirate and washings were collected in separate tubes. Then the radioactivity of all specimens was determined using a gamma counter.

From the counts, the percentage radioactivity of the CPU was calculated as below:

$$\%\ \text{Radioactivity on CPU} = \frac{\text{CPU} \times 100}{\text{CPU} + \text{Aspirate} + \text{Lavages} + \text{Syringe}}$$

The radioactivity reflected the number of SMCs. For further details, please refer to appendix 3.

Cell Viability

Viability of seeded cells were assessed using Alamar blue™ (Serotec Ltd., Kidlington, Oxford, UK) assay, as per our previous protocol(281).

Protocol (Appendix 4).

1. Dilute Alamar Blue™ (Serotec Ltd., Kidlington, Oxford, UK) 1:10 with cell culture medium.
2. CPU segments seeded with SMCs inserted into wells of a 24-well plate
3. Dilute Alamar Blue solution added to cell-containing grafts and control wells.
4. Cover of 24-well plate re-placed and whole thing placed in 37°C / 5% CO_2 incubator.
5. After four hours, 100µl of Alamar Blue solution from each well aspirated and place into well of 96-well plate.

6. Place 96-well plate into spectrophotometer and absorbance read spectroscopically at wavelengths of 570 and 630 nm (Labsystems Multiscan MS visible spectrophotometer).
7. Absorbance is compared to controls without any cells on at all.
8. Values can be taken at one fixed time point or over a time course with serial time points.

Segments were also subjected to scanning electron microscopy (SEM) for the presence and morphological appearance of attached cells.

Data Analysis and Statistical Methods

Experiments were repeated six times. Data are presented with mean $\pm$ standard deviation (SD). For the cell attachment and viability data, the different groups are compared using 1-way ANOVA test using Bonferroni's Multiple Comparison Test.

5.2.2 Role of Cell Concentration & Attachment Time

Assessment of Cell Attachment

Again lengths of CPU were cut into 1-2 cm pieces, inserted into 2ml syringes with the inner surface facing upwards (away from the syringe plunger) and 0.7mls of radiolabelled SMCs at the following concentrations were added for the following periods of time:

1. $5x10^5$ SMC/ml for 24 hours (1/7).
2. $1x10^5$ SMC/ml for 24 hours (1/7).
3. $1x10^5$ SMC/ml for 48 hours (2/7).
4. $2x10^4$ SMC/ml for 48 hours (2/7).

After the specified period SMC solution was aspirated and the CPU surface was lavaged 3 times with PBS. All CPU grafts, aspirates and lavages were measured for radioactivity in a gamma counter.

Cell Viability

Cell viability was assessed spectroscopically after 4 hours contact with 10% Alamar Blue – see appendix 4 for further details.

Phosphoimaging

The CPU segments with radiolabelled SMCs were fixed in acetone, then placed on phosphor storage plates (europium iodide coated) and exposed for 10 days. After exposure the latent image was converted to a quantitative digital image (radioluminograph - RLG) using a 'Storm' phosphor storage plate reader housing a helium-neon laser (Amersham UK).

RLG Image analysis was performed using ImageQuant software (Amersham UK) Regions of interest were drawn and median (mean) counts per pixel calculated.

Data Analysis and Statistical Methods

Experiments were repeated six times. Data are presented with mean $\pm$ standard deviation (SD). For the cell attachment and viability data, the different groups are compared using 1-way ANOVA test using Bonferroni's Multiple Comparison Test.

5.3 Results

5.3.1 Role of Cell Attachment Peptides

Assessment of Cell Attachment

The impact of the various attachment factors on cell attachment are summarised in Table 5.1 and displayed in Figure 5.1. FEPP+ was significantly better than the native

CPU ($p<0.01$) in terms of SMC attachment, with a mean of 31.5±5.9% cell attachment compared to 20.7±4.6% for the Native graft as calculated by the radiolabelling method.

Cell Viability

Alamar blue at 4 hours showed that every attachment factor except FN resulted in significantly higher ($p<0.01$) metabolic activity compared to Native (Figure 5.2).

Attachment Factor	Cell Attachment: %	P value
Native	20.7 ± 4.6	N/A
FEPP+	31.5 ± 5.9	**p<0.01**
SFN	29.4 ± 3.5	p>0.05
FEPP	25.1 ± 4.1	p>0.05
RGD	23.7 ± 2.1	p>0.05
Collagen	22.0 ± 3.4	p>0.05
FN	18.5 ± 5.9	p>0.05

Table 5.1: Smooth Muscle Cell Attachment with Various Attachment Factors.

Mean ± standard deviation. Only FEPP+ significantly enhances cell attachment compared to Native. P-values are comparison with Native: Bonferroni's Multiple Comparison Test. N=6

Key: FN=Fibronectin; SFN=Superfibronectin; FEPP=Fibronectin-like Engineered Polymer Protein; FEPP+=Fibronectin-like Engineered Polymer Protein Plus; RGD=Arg-Gly-Asp.

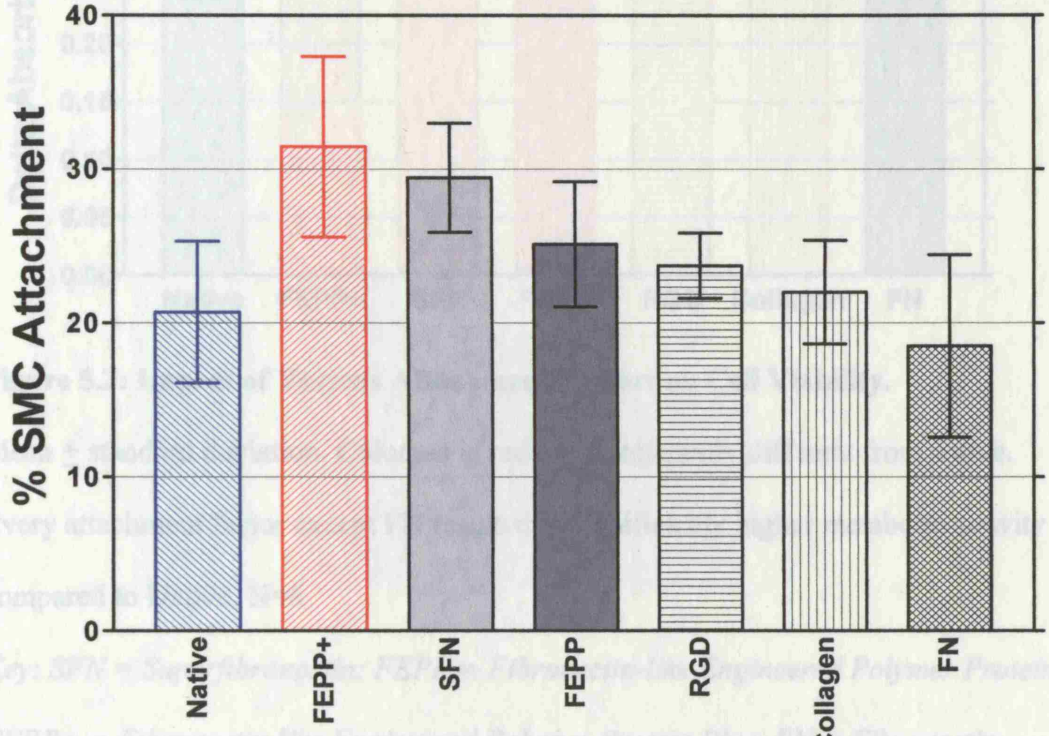

Figure 5.1: Smooth Muscle Cell Attachment with Various Attachment Factors.

Mean ± standard deviation. Red columns are significantly different from native. See Table 5.1 for statistics. Only FEPP+ significantly enhances cell attachment compared to Native. N=6

Key: FN=Fibronectin; SFN=Superfibronectin; FEPP=Fibronectin-like Engineered Polymer Protein; FEPP+=Fibronectin-like Engineered Polymer Protein Plus; RGD=Arg-Gly-Asp.

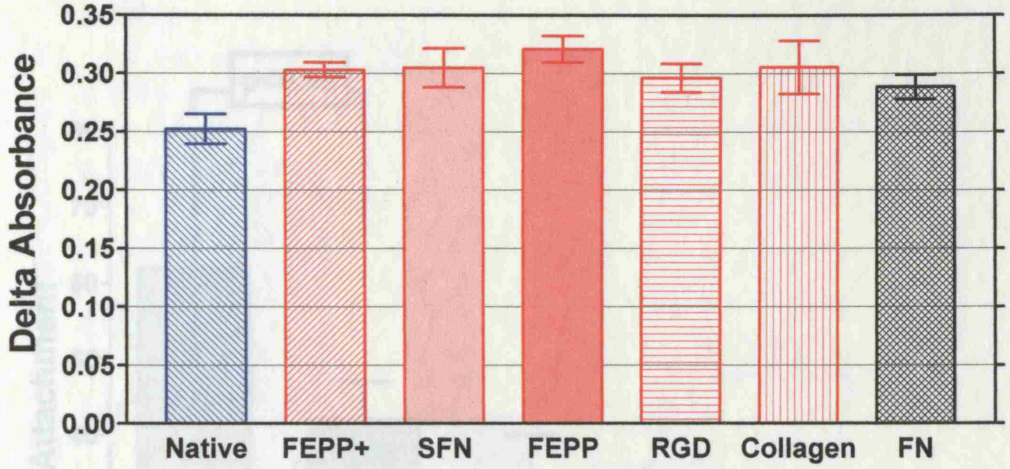

Figure 5.2: Impact of Various Attachment Factors on Cell Viability.

Mean ± standard deviation. Columns in red are significantly different from native. Every attachment factor except FN resulted in significantly higher metabolic activity compared to Native. N=6

Key: SFN = Superfibronectin; FEPP = Fibronectin-like Engineered Polymer Protein; FEPP+ = Fibronectin-like Engineered Polymer Protein Plus; FN = Fibronectin; RGD = Arg-Gly-Asp.

5.3.2 Role of Cell Concentration & Attachment Time

Assessment of Cell Attachment

The impact of the various cell concentrations for different periods of time on cell attachment are summarised in Figure 5.3.

One day (1/7) at $5x10^5$ (62±17.9%) resulted in a significantly increased proportion of cell attachment than 1/7 at $1x10^5$ (42.2±4.9%): $p<0.05$ (1-way analysis of variance with Bonferroni's comparison of all columns).

Other comparisons showed no significant difference between:

1/7 at $1x10^5$ (42.2±4.9%) and 2/7 at $1x10^5$ (33.8±6.8%).

2/7 at $1x10^5$ (33.8±6.8%) and 2/7 at $1x10^4$ (28.2±5.6%).

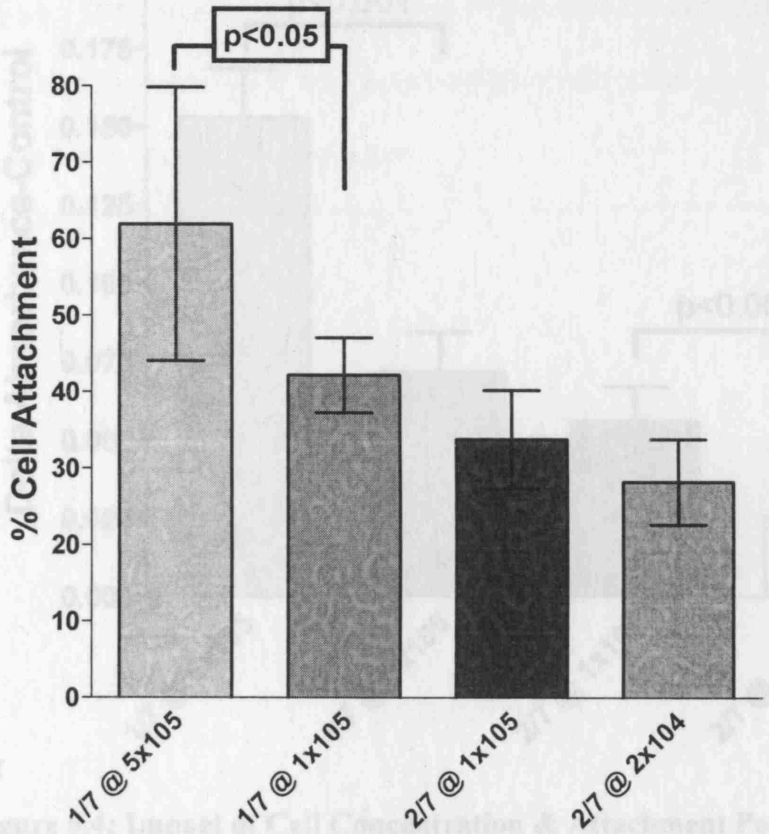

Figure 5.3: Smooth Muscle Cell Attachment with Different Cell Concentrations and Time Periods.

Values are mean ± standard deviation. Higher initial cell concentration resulted in higher proportional cell attachment. N=6

Key: 1/7=1 day; 2/7=2 days; $2x10^4$, $1x10^5$ & $5x10^5$ =SMC concentration/ml.

Cell Viability

The viability data mirrored the cell attachment data as shown in Figure 5.4, with 1/7 at $5x10^5$ showing a significantly higher viability reading compared to 1/7 at $1x10^5$: $p<0.001$ (1-way analysis of variance with Bonferroni's comparison of all columns). 2/7 at $1x10^5$ also had a significantly higher viability reading compared to 2/7 at $2x10^4$: $p<0.001$. There was no significant difference between 2/7 and 1/7 at $1x10^5$.

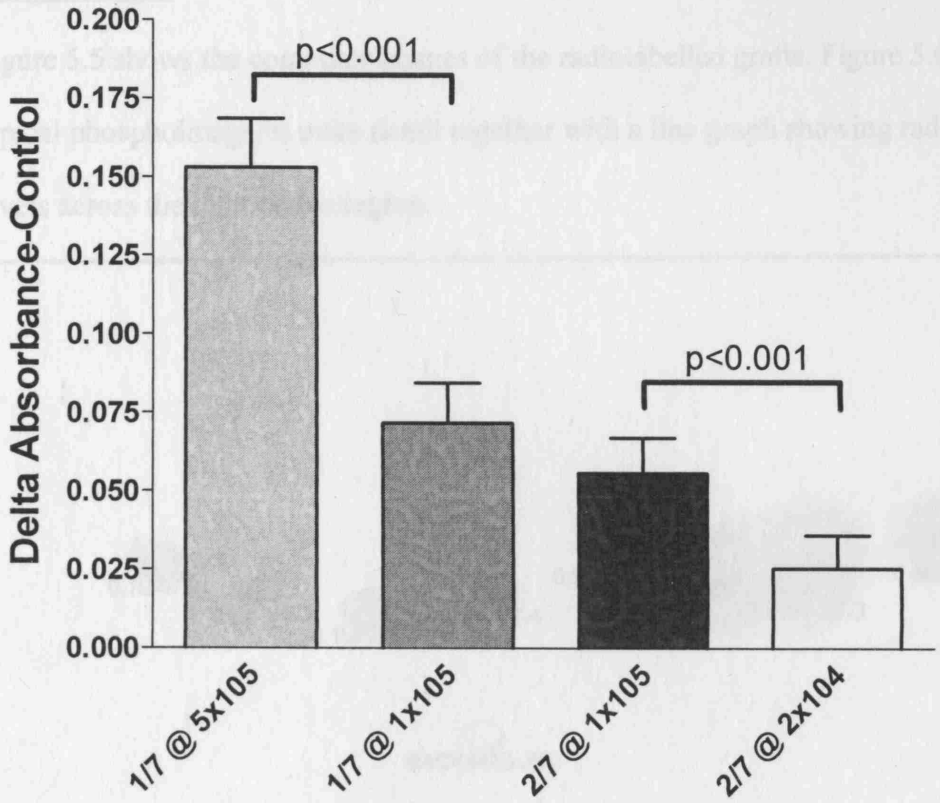

Figure 5.4: Impact of Cell Concentration & Attachment Period on Cell Viability

Values are mean ± standard deviation of delta absorbance with control value subtracted from it. Higher initial cell concentration resulted in higher cell metabolic activity. N=6

Key: 1/7=1 day; 2/7=2 days; $2x10^4$, $1x10^5$ & $5x10^5$ =SMC concentration/ml.

Phosphoimages

Figure 5.5 shows the computer images of the radiolabelled grafts. Figure 5.6 shows a typical phosphoimage in more detail together with a line graph showing radioactivity levels across the radioactive region.

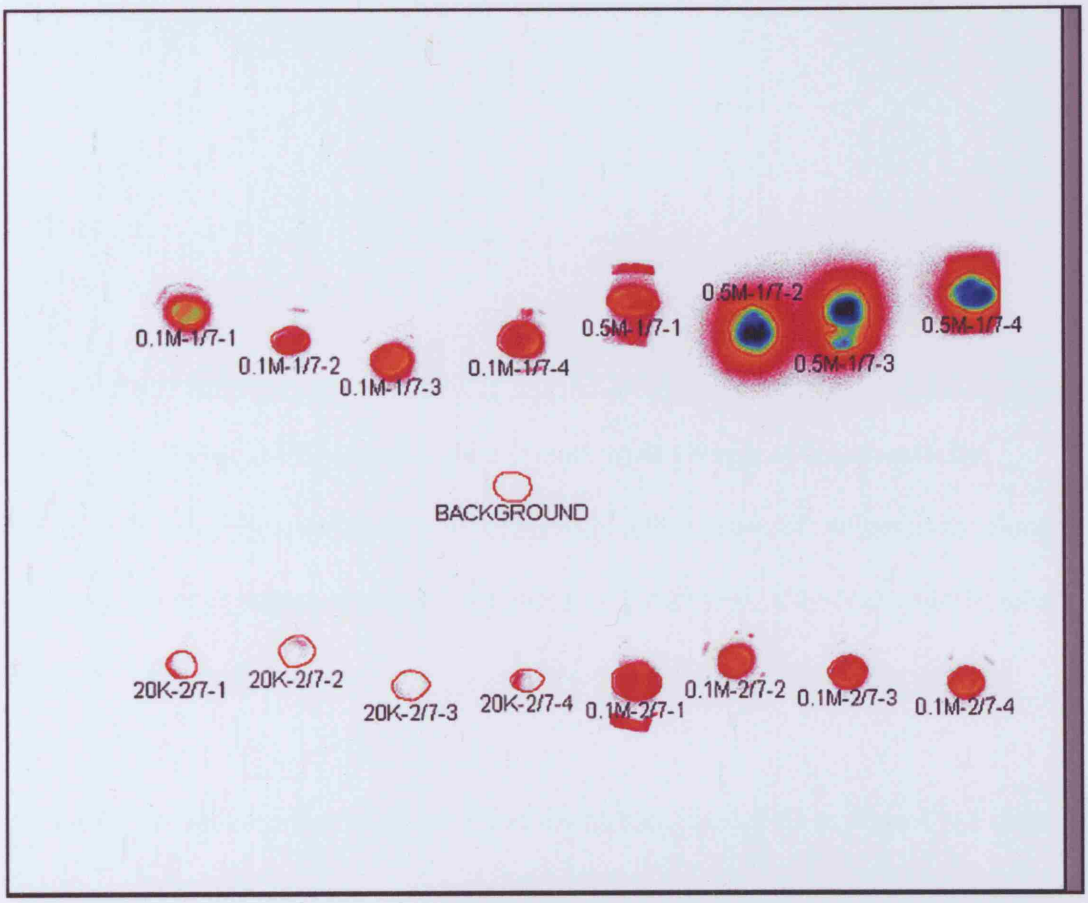

Figure 5.5: Phosphoimages of Grafts with Different Cell Concentrations over Different Time Periods.

Key: M = million; K = thousand; 1/7 = 1 day; 2/7 = 2 days.

Colours represent varying concentrations of radiolabelled cells.

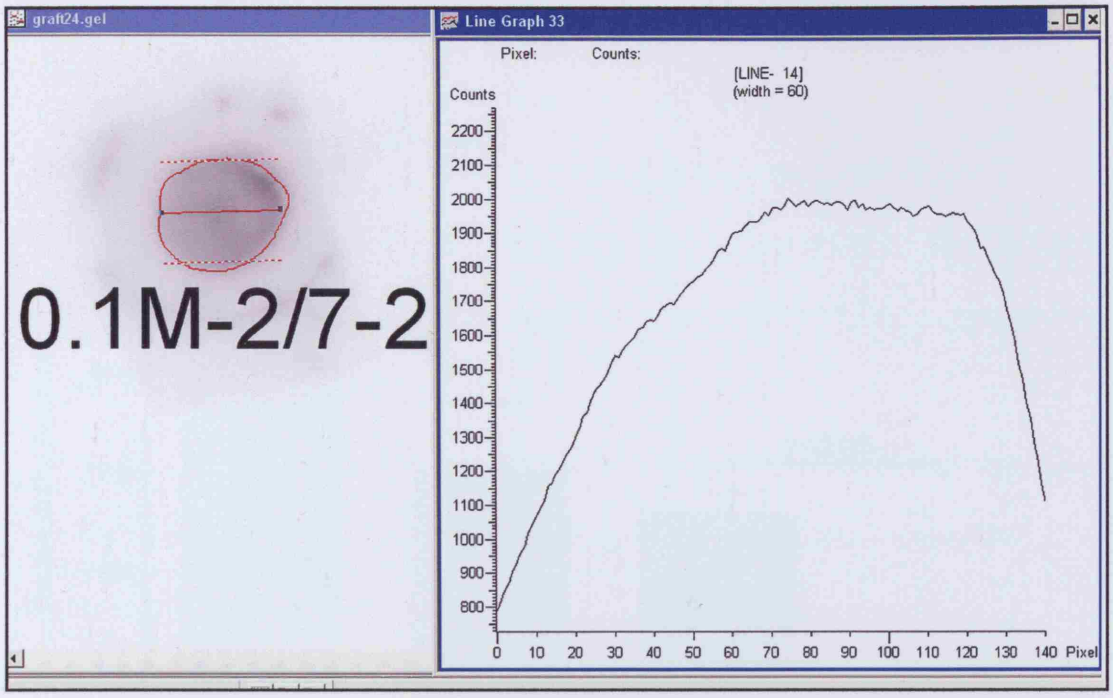

Figure 5.6: Typical Phosphoimage of Graft with Graph of Radioactivity.

To the left is the phosphoimage and to the right a line graph of radioactivity along the width of the graft representing concentration of radiolabelled smooth muscle cells.

Key: M = millions of smooth muscle cells/ millilitre; 2/7 = 2 days.

When the phosphoimages were analysed for radioactive levels as shown in Figure 5.7 there was a significant difference between 1 day at $5x10^5$ and $1x10^5$ SMCs/ml ($p<0.01$ - 1-way analysis of variance with Bonferroni's comparison of all columns) and between 2 days at $1x10^5$ and $2x10^4$ SMCs/ml ($p<0.05$). There was no significant difference between $1x10^5$ SMCs/ml at 1or 2 days.

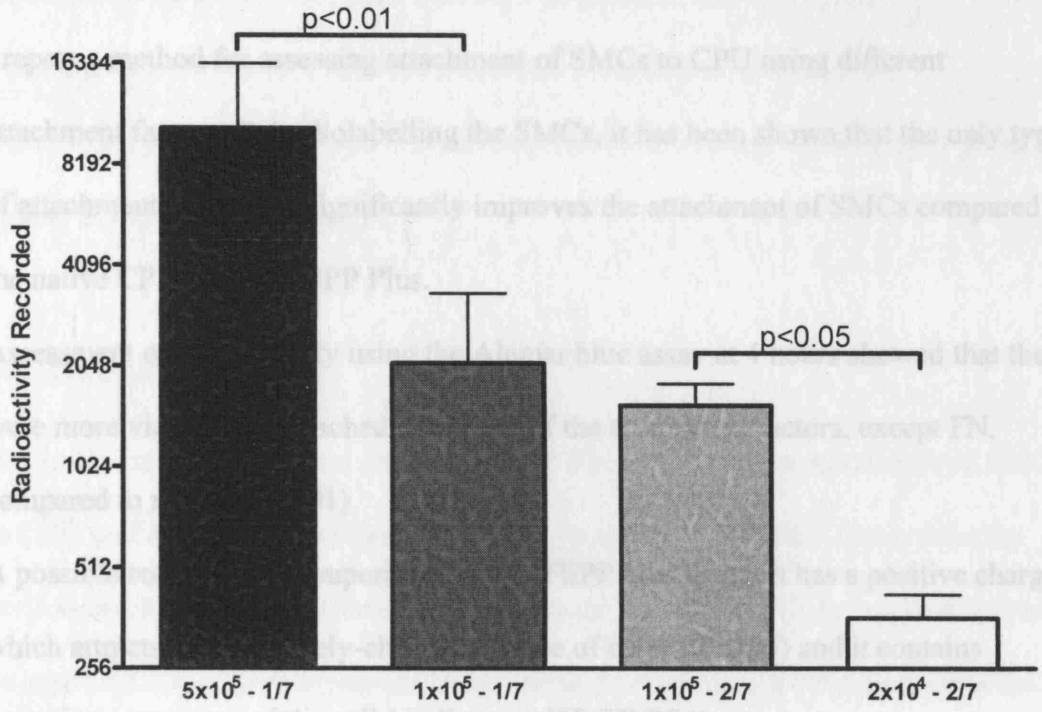

Figure 5.7: Analysis of Phosphoimages of Grafts with Different Cell Concentrations and Attachment Periods

Values are mean ± standard deviation. Higher initial cell concentration resulted in higher measured radioactivity levels. N=6

Key: 1/7=1 day; 2/7=2 days; $2x10^4$, $1x10^5$ & $5x10^5$ = SMC concentration/ml.

5.4 Conclusion

I report a method for assessing attachment of SMCs to CPU using different attachment factors. By radiolabelling the SMCs, it has been shown that the only type of attachment factor that significantly improves the attachment of SMCs compared to the native CPU graft is FEPP Plus.

Assessment of cell viability using the Alamar blue assay at 4 hours showed that there were more viable cells attached using any of the attachment factors, except FN, compared to native ($p<0.01$).

A possible reason for the superiority of the FEPP Plus is that it has a positive charge, which attracts the negatively-charged surface of cells(282;283) and it contains repeating sequences of the cell-binding motif RGD(284).

Experiments on seeding density suggest that the higher the initial seeding concentration the higher the proportion of cells that attach. This was demonstrated by comparing the data from $5x10^5$ and $1x10^5$ SMC/cm^2 which was significantly different. Although the comparison of $1x10^5$ and $2x10^4$ over 2 days showed no significant difference, there was a trend towards enhanced attachment at the higher cell concentration. This may be because cells contacting each other utilise cell attachment factors which can also be used for attachment to the CPU graft. Indeed with endothelial cells seeded onto grafts of PTFE, supraconfluent levels in a process known as 'sodding' are needed in order to show an impact on cell coverage and graft patency(121;285).

There was no significant difference between allowing 1 or 2 days for attachment to occur as demonstrated by the data for $1x10^5$ SMC/cm^2, both in terms of proportionate

(%) attachment, cell viability and the phosphoimage analysis. It seems likely that this was because 24 hours is sufficient for cells to attach to the CPU graft.

The enhanced cell viability measurements and radioactive levels on the phosphoimages with increased cell seeding density are as one would expect given the greater number of cells present.

5.5 Summary

In this chapter I have shown that using FEPP Plus enhances the attachment of SMCs to CPU grafts. Furthermore the higher the concentration of SMCs, the greater the proportionate (%) cell attachment at densities up to $5x10^5$ SMC/cm^2 and there does not appear to be a time-dependent factor to the attachment. All future experiments were carried out at a minimum of $1x10^5$ SMC/cm^2 of graft and cell adhesion was allowed to occur at no more than 1 day.

Having attached the SMCs to the compliant scaffold, the next step involved confirmation of their viability and ability to grow so that a long-term biological layer was possible on the scaffold.

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Chapter 6

CONFIRMATION OF SMOOTH MUSCLE CELL GROWTH ON COMPLIANT POLY(CARBONATE-UREA)URETHANE SCAFFOLD

6.1 Introduction

The previous chapters demonstrated how to optimise the attachment of SMCs to the CPU graft material.

However, a major problem was that there was no way of visualising the development and growth of SMCs on the CPU. This is because the wall of the CPU is too thick for light to penetrate it, thus ruling out conventional light microscopy.

A method therefore had to be developed to keep the SMCs in a sterile environment, allowing access to a supportive growth solution, but at the same time allowing visualisation ("live cell imaging") of the CPU surface by reflection rather than penetration of light.

One solution would be to use a fluorescent microscope, which uses the reflection of light to allow live cell imaging(286). A method needed to be developed for inserting a fluorescent marker into the SMCs, which would keep them sterile, not affect their development significantly and which would stay within the cells. Green Fluorescent Protein (GFP) is a standard method for fluorescent imaging(286;287), which has been used specifically in tissue engineering applications(288).

The method involves the use of retroviral gene vectors(289)with the transduction of target cells. This is done using PT67 embryonic fibroblasts engineered to produce the Murine Leukaemia Retrovirus, which has been engineered to produce the GFP gene. The virus produced can infect and hence insert the GFP gene into cells. However, the virus lacks the packaging instructions required to replicate within the cells they have infected, meaning they are replication incompetent(290). Furthermore, none of the gene products have harmful properties.

In addition to the visualisation of cells, growth was assessed quantitatively over time using the cell viability assay Alamar Blue™ and Pico Green which measures nucleic acid (DNA principally) and therefore indirectly cell numbers.

6.2 Methods and Materials

6.2.1 Transduction of Cells

Approval for the use of transduced human cells was sought and received from the Genetic Modifications Committee of the Royal Free & University College Medical School. This work was done in collaboration with the laboratory of Dr. Giorgio Terenghi, Blond McIndoe Centre, Royal Free & University College Medical School, who kindly supplied the PT67 cells and all equipment used for this experiment.
The method is outlined below

1. SMCs were prepared to 30% confluency in standard T75 flasks, to allow sufficient subsequent division.
    a. All medium was aspirated off.
2. PT67 embryonic fibroblasts were grown in a 32°C incubator.
    a. The supernatant was then aspirated off.
    b. The supernatant was then put through a 45 μm filter to exclude any fibroblasts leaving just the GFP-containing virus.
    c. The PT67 flasks were re-filled with solution.
3. 4.5mls of viral supernatant was added to each T75 flask with SMCs.
    a. The flasks were now put into a 37°C incubator for 15 minutes to allow equilibration.
    b. The flasks were then transferred to a 32°C incubator overnight.

4. The next day the virus-containing medium was aspirated from the flasks and disposed of in 1% hypochlorite solution.
    a. The flasks were re-filled with standard SMC solution and put into a 37°C incubator.
5. The process from steps 2 to 4 was repeated twice more to give 3 transduction cycles.
6. The transduced SMCs containing GFP were then trypsinised from the flasks and resuspended at $2x10^5$ SMCs per ml and put into:
    a. Chamber slides and T25 flasks to see how they grew under normal conditions.
    b. Onto lengths of CPU, which had been cut open longitudinally and laid flat onto an autoclaved glass slide, held in place by sterile 2/0 vicryl ties. The whole of the slide was then placed in a Petri dish and filled with standard SMC solution.
7. The flasks and Petri dish had their medium changed twice weekly, with images taken from a fluorescent microscope whenever the medium was changed.

6.2.2 Quantitative Measurement – Alamar Blue & Pico Green

The protocol for the quantitative assessment of SMC growth on the CPU scaffold is outlined below

1. CPU scaffolds from a 5mm graft were cut into flat pieces of 10mm diameter and placed in the wells of a 24-well plate. They were held down at the bottom of the well by cut pieces of autoclaved silicone tubing which had an external diameter of 14mm, internal diameter of 10mm and were 10mm in height.

2. SMCs from UC were extracted and grown to passage 6 after which they were trypsinised.
3. The SMCs were pipetted onto the CPU grafts in the wells at a seeding density of $3x10^5$ SMCs/cm^2.
4. The SMCs were left on the grafts overnight to settle.
5. Grafts which had their medium changed on days 1 and 4 unless their cellularity was being assessed.
6. Grafts having their cellularity assessed initially had cell viability assessed as follows:
    a. All the medium was aspirated followed by 3 lavages using sterile PBS.
    b. 1.25 ml of 10% Alamar Blue solution was added - as per the protocol of Appendix 4 – to the grafts in the wells.
    c. After 4 hours the assay was measured – as per protocol of Appendix 4.
7. After the Alamar Blue Assay, nucleic acid was assayed using Pico Green® dye (Molecular Probes Co., OR, USA) as follows (see Appendix 5):
    a. All the Alamar Blue solution was aspirated followed by 3 lavages with sterile PBS.
    b. Add 1ml of clear trypsin solution to the grafts in the wells to dislodge any cells.
    c. Incubate at 37°C for 10 minutes with occasional gentle agitation.
    d. Aspirate all trypsin solution and wash 3 times with sterile PBS, collecting all washings in a 1.8ml PCR tube.
    e. Aspirate and expel all collected trypsin and PBS washings through a 23G needle attached to a 2ml syringe 3 times.
    f. Freeze at -4°C and then thaw a few days later.

g. Sonicate the PCR tubes for 5 minutes.

h. Mix contents thoroughly.

i. A set of standard controls consisting of serially diluted calf thymus DNA were prepared.

j. In 96-well plates: 100 µl aliquots of samples and controls were mixed with 100 µl 1:400 (v/v) dilution of Pico Green in 1x TE.

k. The solutions were then incubated for 5 minutes in the dark.

l. Measurements of emissions at 538nm after excitation at 485nm were read in a Fluoroskan Ascent FL, Thermo Labsystems.

Data Analysis and Statistical Methods

Experiments were repeated six times. Data are presented with mean ± standard deviation (SD). For both the Pico Green and Alamar Blue, the data for each two successive time points are compared using two-tailed T-test.

6.3 Results

6.3.1 Transduction of Cells

The images taken from a fluorescent microscope using a digital camera in figure 6.1 show that the SMCs in flasks and chamber slides were successfully transduced with GFP. Similarly, the images in figure 6.2 show that the GFP-labelled SMCs have successfully attached and grown onto the CPU 4 days after seeding.

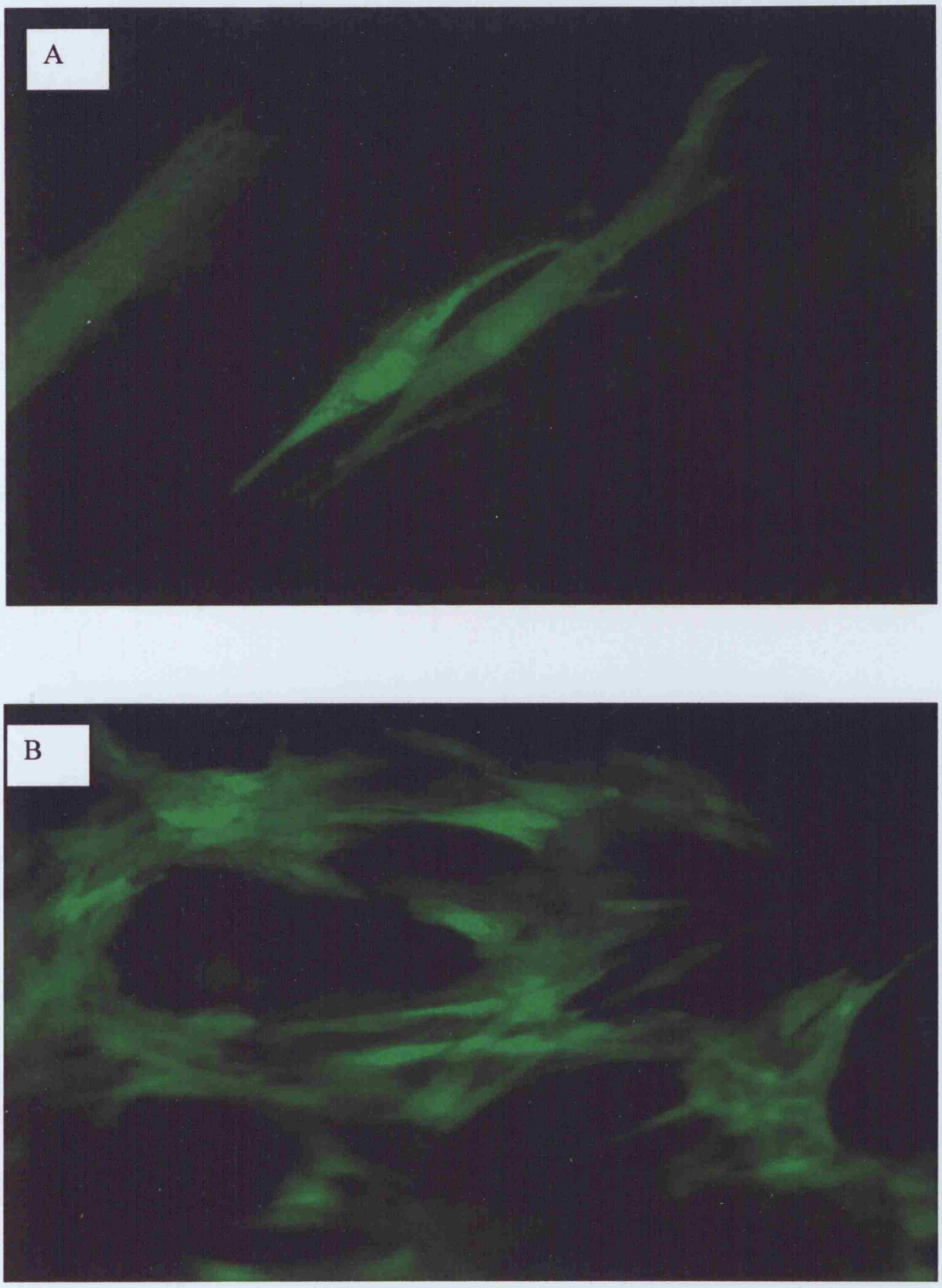

Figure 6.1: Smooth Muscle Cells Transduced with GFP

(A): x40 magnification; (B): x10 magnification

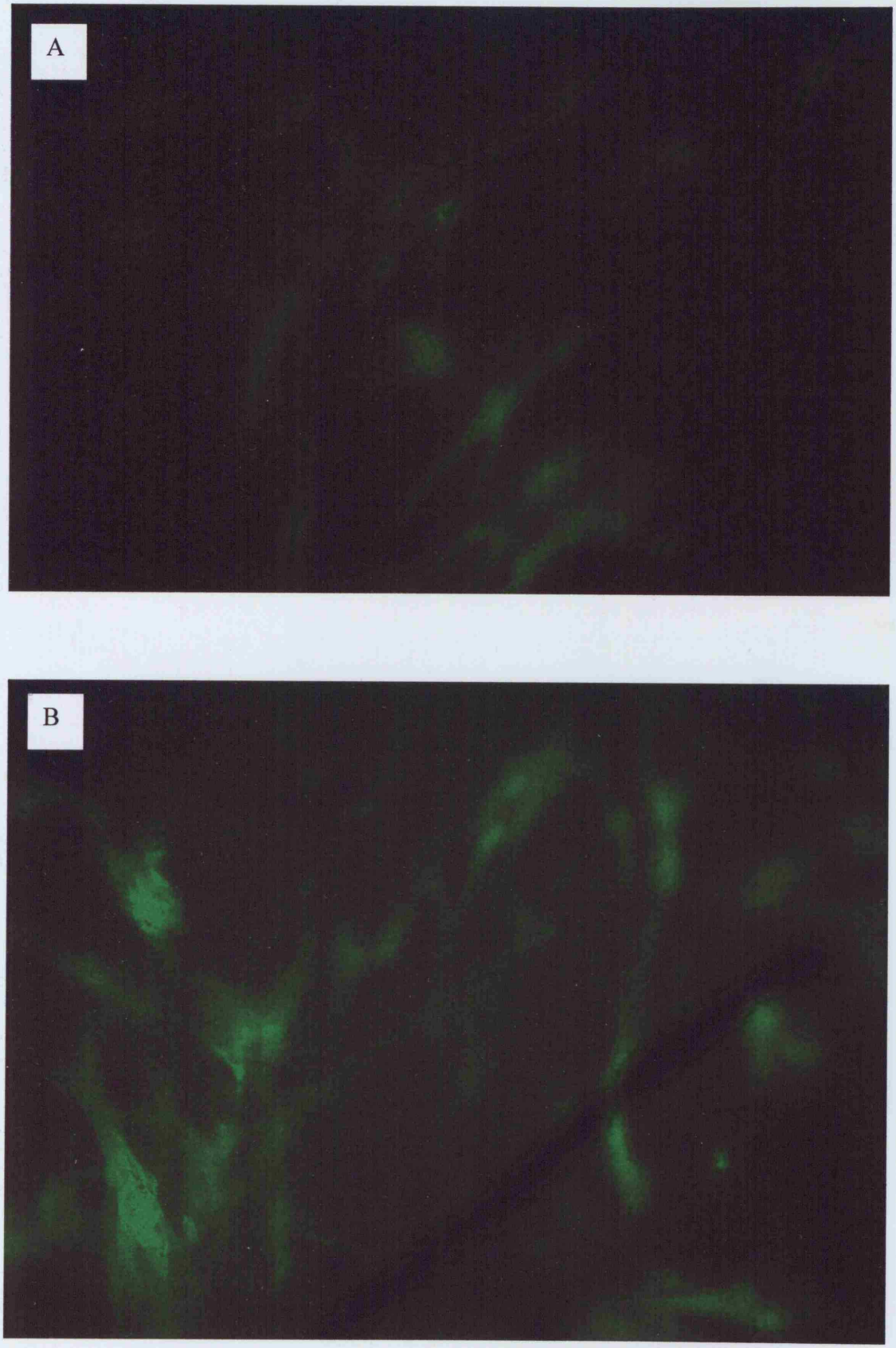

Figure 6.2: Smooth Muscle Cells Transduced with GFP on CPU

(A) x10 magnification; (B): x20 magnification

6.3.2 Quantitative Measurement – Alamar Blue & Pico Green

Figures 6.3(a) details the changes in the cell viability assay Alamar blue at the three time points whereas Figure 6.3(b) graphs the Pico Green assay of nucleic acid at the three time points. The difference in values between day 1 and 4 was significant ($p<0.05$, two-tailed T-Test) whereas it was not significant between days 4 and 7.

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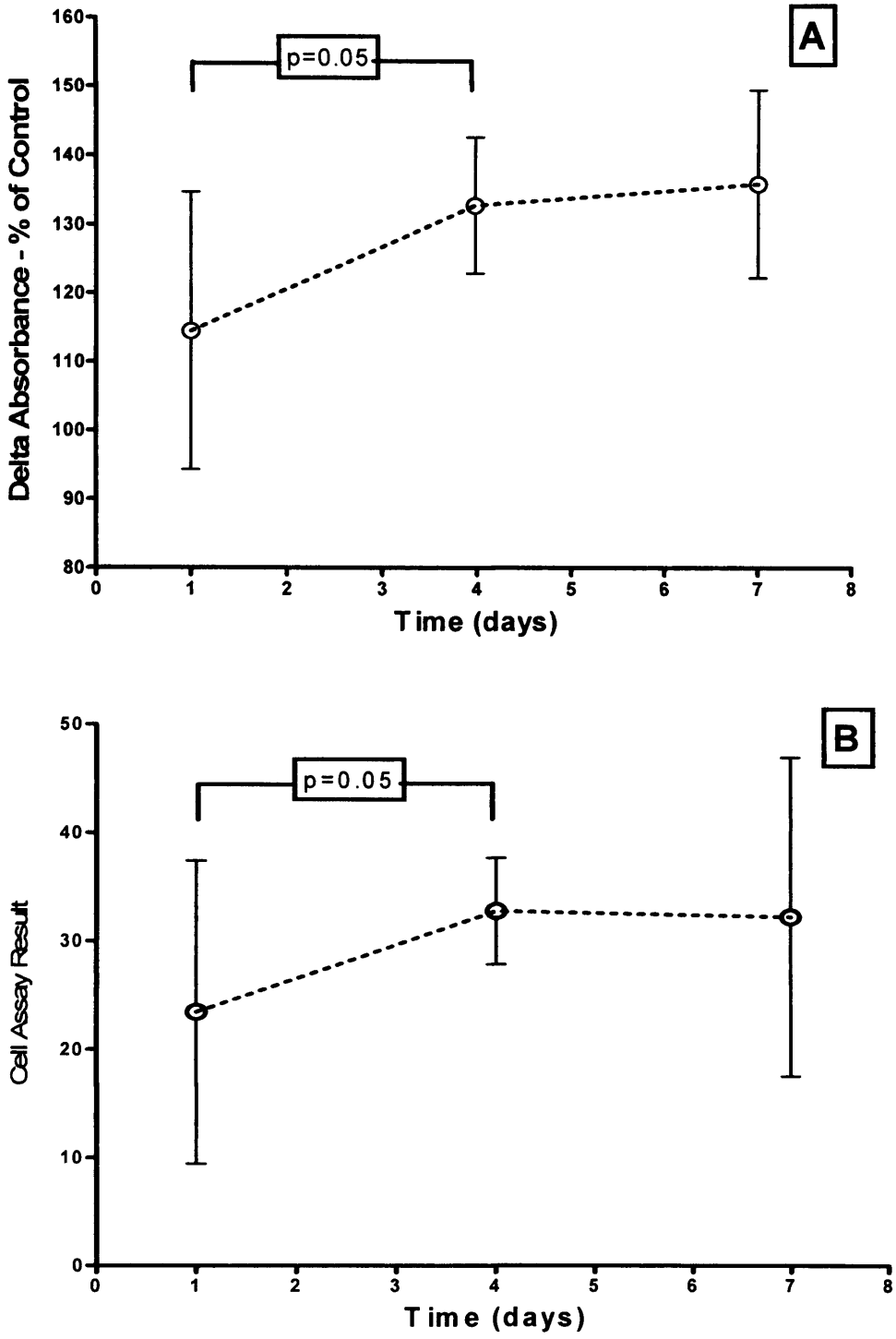


Figure 6.3: Smooth Muscle Cell Growth on CPU over Time

(A) Alamar Blue; (B) Pico Green. Increased levels of metabolically active cells (Alamar Blue) and nucleic acid (Pico Green) with time suggesting cell replication.

N=6

Key: SMC = Smooth Muscle Cells; Values are Mean ± Standard Deviation

6.4 Conclusion

This chapter describes a methodology for qualitatively visualising and thus proving that SMCs do attach and grow onto the inner surface of the CPU graft. This overcame the nagging doubt of the previous experiments that the radioactivity detected after lining CPU with radioactive SMCs was not due to the SMCs but the radioactive Indium leaching out of the cells. Unfortunately, this experiment using GFP-labelled SMCs is not a quantitative assessment of percentage coverage of the CPU surface because not all SMCs are transduced, and so one is only ever visualising a percentage of the SMCs on the graft.

The data in this chapter also provide quantitative evidence of the proliferation of SMCs on the CPU scaffold using assays for both viable cells and nucleic acid content. As can be seen from the Figures 6.3 (a) and (b) most of the proliferation occurs between days 1 and 4 with some plateauing out between days 4-7. This is reflected in the significant difference in values between days 1 and 4 but not between days 4 and 7. The probable explanation for this is that the cells are reaching confluency and so are inhibited from further growth. Perhaps a lower initial seeding density would have allowed more growth to be shown in the second half of the week.

6.5 Summary

This chapter shows that it is possible to overcome the limitations of the CPU graft in terms of live cell imaging by transduction of GFP into the target cells: in this case SMCs. Not only is this useful for *in vitro* work, but also would be invaluable in any future *in vivo* work in order to differentiate host cells from transplanted cells on the graft. This is otherwise very difficult if not impossible.

Evidence of SMC proliferation on the CPU scaffold is shown by using assays to assess the amount of viable cells (Alamar Blue) and also nucleic acid (Pico Green). These assays show that cell numbers do seem to increase with most of that increase being within the first few days of seeding onto the scaffold.

The next step in the development of the hybrid graft required study of the graft *in vitro* under the conditions a blood vessel would experience *in vivo*.

Chapter 7

DEVELOPMENT OF A PULSATILE FLOW CIRCUIT FOR LONG-TERM CULTURE

7.1 Introduction

One of the key difficulties in tissue engineering is to culture the developing tissue in its 3-dimensional (3D) form over a long period of time. The major problems include supply of adequate nutrients with removal of wastes and maintenance of sterility. Tissue engineers have used bioreactors to grow desired tissues in a 3D form and used flow circuits to provide the requisite mechanical inputs.

Pulsatile blood flow results in a mechanical stimulation composed of hydrostatic pressure and two major vectorial elements: tangential shear stress and circumferential stretch-relaxation(291;292). Endothelial cells contact the flowing blood and so are exposed to all three forces, whereas for smooth muscle cells the most important is the cyclic strain caused by stretch-relaxation(292).

Shear stress is the tangential stress applied across the endothelial cell surface due to the bulk flow of blood(292). The effects on endothelial cells of shear stress and cyclic strain are similar and include increased cell proliferation, increased production of ECM molecules like collagen and fibronectin, and changes to the synthesis and secretion of proteins like increased production of tissue plasminogen activator (tPA) and nitric oxide (NO). Interestingly though, the impact on morphology of shear stress is to align ECs parallel to the direction of flow, which is opposite to the impact of cyclic strain which aligns ECs perpendicular to the strain vector(292;293).

Shear stress can also affect SMCs – shear stress which is turbulent(294) or abruptly falls(295) causes increased SMC proliferation but elevation of shear stress in an endothelialised graft was associated with decreased SMC proliferation(296;297). SMCs have been shown to align perpendicular to the direction of flow in a dose and time dependent manner(298), although this has not been a universal finding(299).

Cyclic strain through stretch-relaxation has been shown to orientate SMCs perpendicular to the direction of the stretch(300-303), generally to stimulate cell proliferation(304;305) though there are some conflicting reports(292), modulate cell phenotype of SMCs from a synthetic to a more contractile type(291;304;306) and even stimulate the differentiation of progenitor cells towards a SMC phenotype(307). Indeed cyclic strain has also been associated with increased production of ECM components like collagen and elastin(305;308;309).
Culturing tissues and particularly tissue-engineered blood vessels within a flow circuit has been shown to improve the 3D structure of the vessel and therefore key mechanical properties like burst pressure(188;310).

This chapter will initially review the use of flow circuits with Tissue-Engineered Blood Vessels: specifically looking at the role of flow circuits in developing TEBV and assessing their mechanical and biological properties. Thereafter there will be a description and testing of the physiological flow circuit developed and used for tissue engineering work carried out in this thesis.

7.1.1 Flow Circuits

Flow circuits consist of a pump, tissue-containing area, tubing and medium flowing through it. They must have a heater and pH-buffering system and have the capacity to exchange gases and, depending on the culture period, nutrients for wastes. Furthermore they usually have some sort of monitoring system to measure parameters like pressure and flow. They can be used for developing tissues, assessing tissues both in biological and mechanical terms.

Traditional pumps – as for example used clinically for heart bypass and dialysis machines - can be either centrifugal or roller. Centrifugal pumps consist of a fanned impeller or a nest of smooth plastic cones that sit inside a plastic housing. The impellers or cones are magnetically coupled with an electric motor and, when rotated rapidly, generate a pressure differential that causes the movement of fluid. On the other hand a roller pump includes a length of tubing, located inside a curved raceway. The raceway lies at the outer perimeter of rollers mounted on the ends of rotating arms (usually two, 180 degrees apart). The system is arranged so that one roller is compressing the tubing at all times. Flow of blood is induced by compressing the tubing, thereby pushing the blood ahead of the moving roller. Flow rate depends upon the size of the tubing, length of the track, and rotation rate of the rollers (revolutions per minute). For a given pump and type and size of tubing, flow is proportional to pump speed (in revolutions per minute)(311).

An alternative pumping system has been described using a mechanical ventilator to drive air into a fluid-filled circuit creating pulsatile laminar flow with physiological variables of flow, blood and pulse pressure(312-315). It has been used to successfully tissue engineer myofibroblasts and ECs on bioabsorbable scaffolds into vascular grafts in a 'biomimetic' environment(316). This innovative design has however been confined to a limited number of researchers and is relatively complex compared to more traditional systems.

The advantage of centrifugal pumps is that when the fluid is blood, there is less cell destruction. However, roller pumps are simpler, cheaper, produce a predictable output, and are capable of generating greater degrees of pulsatile flow(311).

7.1.2 Bioreactors

Bioreactors are devices which assist in the development of new tissue *in vitro* by culturing isolated cells seeded on 3-dimensional scaffolds in an environment with appropriate stimuli(317). Spinner flasks and rotating wall vessel reactors have been used. The spinner flask is the simplest bioreactor design consisting essentially of cell-seeded scaffolds attached to needles hanging from the cover of a flask. The flask is filled with medium sufficient to cover the scaffolds and the medium is mixed with a magnetic stir bar. The rotating wall vessel reactor simulates microgravity effects by using centrifugal forces to oppose those of gravity. The commonest design involves placing the scaffold between two concentric cylinders with gas exchange through the stationary inner cylinder whilst the outer cylinder rotates in a controllable fashion(317).

Rotating wall bioreactors have shown higher seeding efficiency and less cell damage(318). From a blood vessel viewpoint the early bioreactors were simply moulds with an inner mandrel which allowed the 'vessel' to develop with little external cues(190;203).

Since that time increasingly complex systems have been developed but usually based around the flow-perfusion bioreactor system. This involves a pump to perfuse medium continuously through the developing tissue and thus has several advantages over previous systems: principally enhanced delivery of nutrients and the provision of mechanical stimulation to the cells in the form of shear stress and cyclic strain. Bancroft determined four key properties of a successful design(317) as described below.

1. Deliver flow through the scaffold and not around it.

2. Repeatable, consistent and controllable flow rate.

3. Sterilisable and able to remain sterile.

4. Reasonable to operate: not too complex.

One model made of glass contained a stir bar for even distribution of medium when placed on a stirplate, a lid for gas exchange and a rubber membrane for medium exchange. The side arms allowed silicone tubing to be threaded through the bioreactor to act as a physical mandrel around which a vessel scaffold could be cast and allowed connection to a flow circuit(188;310).

Recent developments that are applicable to blood vessel development include a laminar flow chamber connected to a pump in which the flow chamber was linked directly to a 'nutrition' chamber which nourished the cells from the side not facing the main flow(319). The 'multicue bioreactor' uses a computer-controlled pulsatile pump to apply pressure and strain conditions to a cell-seeded scaffold. It is even able to replicate the dicrotic notch in its pressure waves, apply longitudinal strain and has two-way perfusion(320).

The ability to have longitudinal strain has also been incorporated by other bioreactor designs on the basis that arterial growth in embryonic vessel development is associated with longitudinal strain. Mironov et al developed a system comprising a chamber and two peristaltic pumps to allow separate perfusion of the inside and outside of the developing vessel. The longitudinal strain is created by a sliding frame attached to one end of the developing vessel and can vary from 0-200%(321). A more basic version of this system was used to increase the length of juvenile porcine carotid arterial segments by 40%. Gradual longitudinal strain was applied in a bioreactor-type chamber whilst the artery was exposed to pulsatile flow of medium through the lumen(322).

A more elaborate approach which combines cell seeding and perfusion in single bioreactor design has been developed. The bioreactor consists of 3 separate chambers: an air chamber, cell medium chamber and cell-seeding and perfusion chamber. The latter allows injection of cells onto a scaffold and rotation of that scaffold for even cell distribution. The air chamber is connected to a respirator pump and so moves medium in a pulsatile fashion from the medium chamber through the cell-seeded scaffold in the perfusion chamber. The system allows dynamic mechanical conditioning of the developing conduit in a closed-loop environment that facilitates maintenance of sterility(314).

7.1.3 Uses of Flow Circuits & Bioreactors

The main functions of flow circuits with or without bioreactors are investigating the impact of mechanical forces on cells and tissues, culturing tissues especially in three dimensions and the assessment of physical and biological properties of tissues.

A) Investigating Impact of Mechanical Forces

Flow circuits can be used to investigate the impact of fluid shear stress and also cyclic strain - if the flow is pulsatile - on cells and tissues. As mentioned in the introduction to this chapter shear stress and cyclic strain impact upon SMCs and ECs. Flow circuits have been used to delineate the impact of shear stress on cell alignment(298;299) and gene expression(323).

For example, myofibroblasts from human aorta were seeded on polyglycolic acid (PGA) scaffolds placed in rigid frames and exposed directly to pulsatile medium flow in a chamber. When compared to a static group, the shear-stress group of seeded scaffolds had much greater ECM production as determined by hydroxyproline assays – a marker of collagen production(319).

A flow circuit with vascular SMCs seeded onto PGA scaffolds in bioreactors - where the scaffolds were separated from the medium by silicone tubing - was used to determine the impact of cyclic strain rather than fluid shear stress at different pulse rates: fetal 165 beats per minute (bpm), adult 90bpm and static. The fetal pulse rate of 165 bpm showed greater collagen production and ECM remodelling(324). This model was used to demonstrate that the vessels grown with pulsatile flow had significantly greater mechanical strength and improved morphology than those without pulsatile flow(188;310).

A simple bioreactor-flow circuit system was used to increase the length of juvenile porcine carotid arterial segments by 40%. Gradual longitudinal strain was applied in a bioreactor-type chamber whilst the artery was exposed to pulsatile flow of medium through the lumen(322).

The impact of pre-conditioning has also been studied using flow circuits. Shear stress pre-conditioning was shown to enhance the retention of ECs when exposed to arterial blood pressure(191;325;326). Similarly decellularised porcine vessels were seeded with endothelial progenitor cells and then placed in a laminar flow bioreactor. Pre-conditioning with a gradually increasing shear stress resulted in greater cell density upon exposure to arterial levels of shear stress(182).

B) Culturing Cells into Tissues

The first attempt to develop a tissue-engineered blood vessel used a primitive bioreactor consisting of a test tube with an inner glass mandrel wherein SMCs contracted a collagen gel. This was surrounded by a Dacron mesh followed by a fibroblast layer and then the lumen was seeded with ECs. This did not allow for any mechanical inputs to be applied.

Another model used sheets of SMCs rolled around a perforated tubular mandrel of PTFE and an acellular membrane made from a dehydrated fibroblast sheet. This construct was then placed into a bioreactor to receive luminal flow of medium for a week before a sheet of fibroblasts was wrapped around the outside and the maturation was continued for 8 weeks. The final stage was luminal seeding of ECs(211).

C) Assessment of Tissues

Whole vessels can be connected to a flow circuit and used to understand the pathogenesis of atherosclerosis. Animal studies are problematic because many factors can't be controlled. Cell and organ culture experiments mean factors are studied in isolation. Within a pulsatile flow circuit, variations of blood pressure, shear stress or cyclic strain can be controlled and either studied in isolation or for pharmacological studies(327;328).

Flow circuits can be used to assess scaffolds for cell retention. This has been done using radiolabelled seeded cells and recording the fall of radioactivity when exposed to arterial flow(118;141;279;329).

One critical parameter that all bypass grafts must have is the ability to resist the constant pressure of the arterial pulse. This means that vessels should not dilate over time to form aneurysms or worse rupture outright. Investigators have tested vessels by increasing the intralumenal pressure until they burst ("burst pressure"). Very simply fluid is forced into the vessel which is clamped distally until the pressure bursts the vessel, the pressure is measured by connecting the flow circuit via a side arm to a pressure monitor(188;190;211).

7.2 Methods & Materials

7.2.1 Development of Novel Flow Circuit & Bioreactor

As mentioned, Bancroft's four key properties of a successful design(317) are:

1. Deliver flow through the scaffold and not around it.

2. Repeatable, consistent and controllable flow rate.

3. Sterilisable and able to remain sterile.

4. Reasonable to operate: not too complex.

A) Bioreactor

The bioreactor designed in-house has gone through various prototypes and can be used for cell seeding, rotation, static and dynamic culture (Figure 7.1). It consists of a transparent Perspex barrel, which can have side-ports drilled into it. These side ports are traversed by threaded luer locks made of polyethylene (Sigma A7677). An airtight seal is achieved by placing a small rubber 'O' ring between the luer lock and the Perspex barrel.

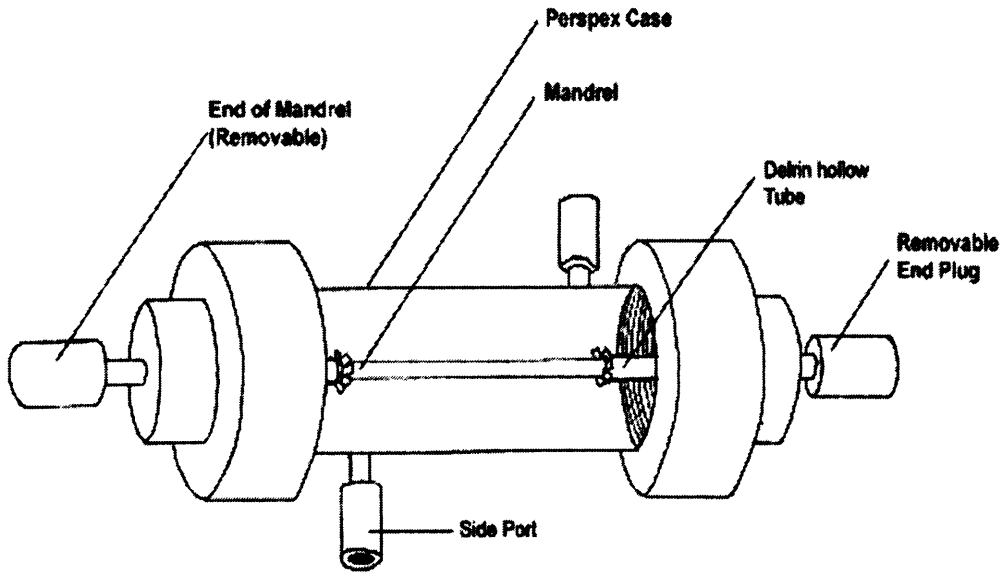


Figure 7.1: Bioreactor

Daigrammatic representation of fully assembled bioreactor.

The caps at either end are made from delrin. A seal is formed between the barrel and delrin end by placing a rubber 'O' ring within the delrin end section which is then screwed onto the Perspex barrel which has a male thread on the outside.

One delrin end is cut as a single section from a solid piece of delrin to minimise threaded seals and cut down possible entry routes for contamination. It has a hollow central tube onto which grafts can be attached. The opposite end is in 3 segments. Firstly there is an inner cap which screws onto the Perspex barrel. This cap has a central hole and its outer end is narrowed and has a male thread. A hollow cylindrical tube of delrin approximately 6 mm in diameter is inserted through the central hole of the inner cap. This end section is secured and made leak-proof by means of a further outer delrin cap which slides over the outside of the central tube and then screws onto the inner cap. A rubber 'O' ring between the three sections completes this assembly and is placed outside the cylindrical tube and between the inner and outer caps. These extra subsections (Figure 7.2) are necessary in order to remove the final graft structure without stretching or twisting. A central stainless steel mandrel passes from end to end. This also has a turned delrin section with an 'O' ring fitted to a bevel towards the outer end which maintains an airtight seal against the inside of the central tube of the end piece of the bioreactor. At the opposite end, the final seal is made with a sealing delrin plug which also has an 'O' ring fitted to make the seal airtight.

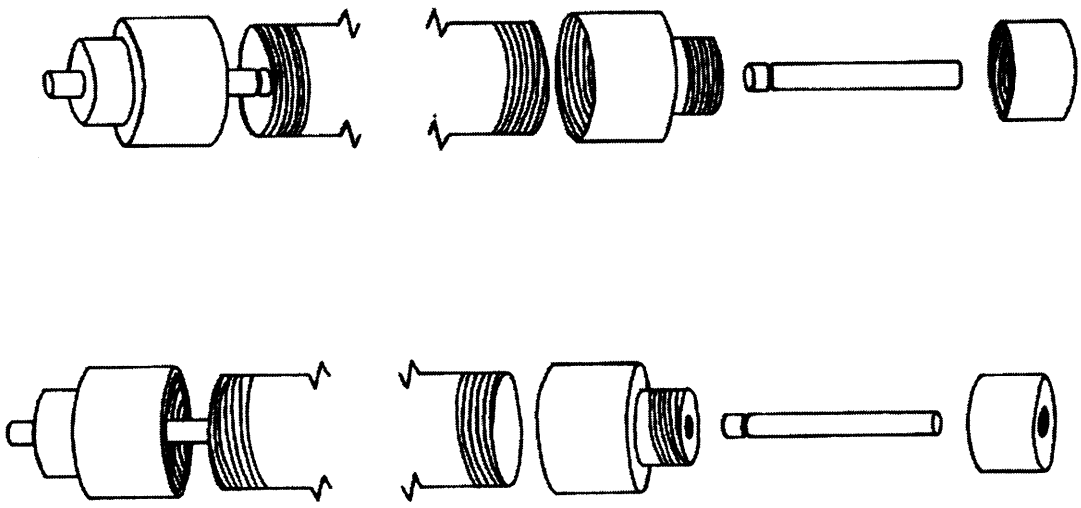


Figure 7.2: Bioreactor Parts

Diagrammatic representation of how the bioreactor parts are separated from each other

The bioreactor can be sterilised using an autoclave at 121°C and is assembled in a sterile flow hood using sterile surgical gloves and an aseptic technique. The hollow cylindrical delrin tubes traversing the caps are designed to hold tubular scaffolds because they have a circumferential indentation on the outside which allows either an 'O' ring to be rolled over the scaffold or a 2/0 silk tie to be secured over the scaffold and onto the indented delrin tube. Once assembled cells can be pipetted in through the ends via the cylindrical tubes or injected through the side ports. Culture medium can also be infused through the side ports and exchanged at regular intervals (Figure 7.3(a), 7.3(b) and 7.4).

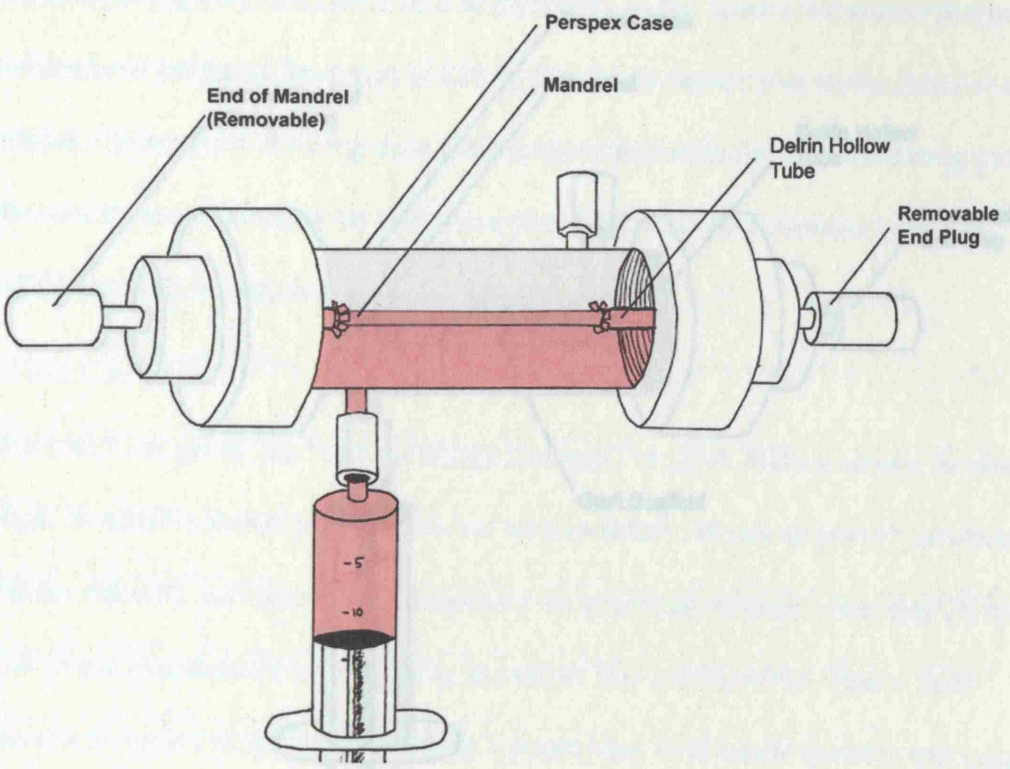


Figure 7.3(a): Infusing Fluid into Bioreactor

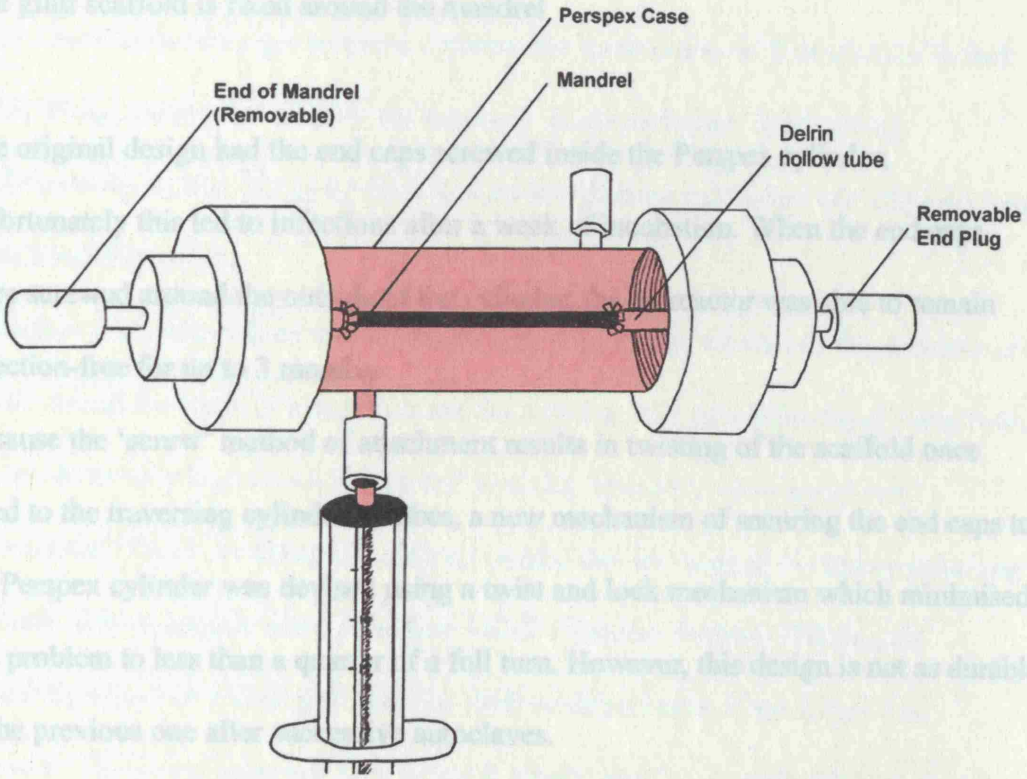


Figure 7.3(b): Bioreactor Filled with Fluid

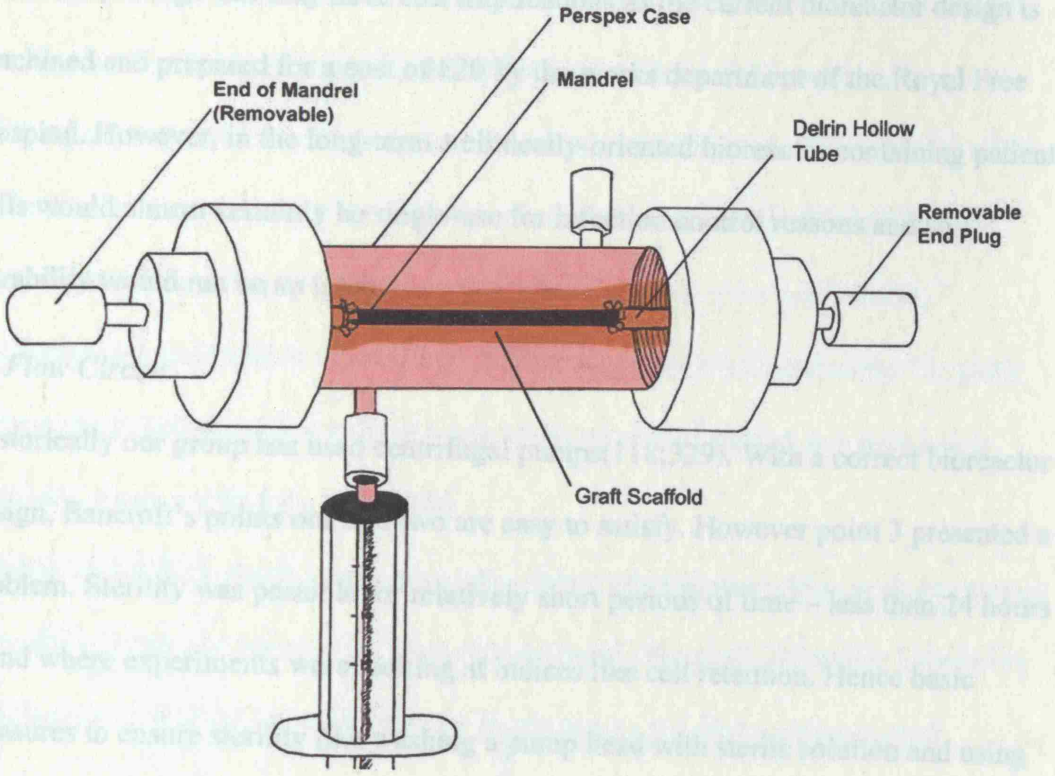


Figure 7.4: Bioreactor with Graft and Filled with Fluid

The graft scaffold is fixed around the mandrel

The original design had the end caps screwed inside the Perspex cylinder; unfortunately this led to infections after a week of incubation. When the end caps were screwed around the outside of the cylinder, the bioreactor was able to remain infection-free for up to 3 months.

Because the 'screw' method of attachment results in twisting of the scaffold once fixed to the traversing cylindrical tubes, a new mechanism of securing the end caps to the Perspex cylinder was devised using a twist and lock mechanism which minimised this problem to less than a quarter of a full turn. However, this design is not as durable as the previous one after successive autoclaves.

Indeed one of the criticisms of the bioreactors is that the Perspex barrels do crack after several autoclave cycles. Ideally a more durable transparent material would be

preferable, though this may have cost implications as the current bioreactor design is machined and prepared for a cost of £20 by the works department of the Royal Free Hospital. However, in the long-term a clinically-oriented bioreactor containing patient cells would almost certainly be single-use for infection control reasons and so durability would not be an issue.

B) Flow Circuit

Historically our group has used centrifugal pumps(118;329). With a correct bioreactor design, Bancroft's points one and two are easy to satisfy. However point 3 presented a problem. Sterility was possible for relatively short periods of time – less than 24 hours – and where experiments were looking at indices like cell retention. Hence basic measures to ensure sterility like washing a pump head with sterile solution and using antibiotics in the medium were sufficient to ensure no significant contamination occurred in the experimental period. However over longer culture periods of days the flow circuit invariably got infected. Options for sterilisation of flow circuits include autoclaving, gamma irradiation and ethylene oxide perfusion. Autoclaving unfortunately melted the pump head and neither gamma irradiation nor ethylene oxide were available on site.

Therefore each experiment would need a new pump head. Given our requirement of a sterile circuit for repetitive experiments, the expense of a new centrifugal pump head was prohibitively high (several hundred pounds). Instead a roller pump was investigated. There are many old dialysis pumps that are surplus to requirements and we were able to acquire some (Gambro AK10 - Gambro Hospal, Huntington, Cambridgeshire) as a kind gift from the dialysis department of the Royal Free Hospital. The pump squeezes flow through tubing which is specifically designed for

this purpose and is medical-grade sterile (Gambro AK90, BL805 – Gambro Hospal, Huntington, Cambridgeshire). This solved point 3 of Bancroft's requirements.

With regard to point 4, these pumps can work continuously for weeks without difficulty, are simple to operate and easy to maintain and service. Given they are designed for life-saving medical treatment; they definitely give a repeatable, consistent and controllable flow rate which can be pulsatile or continuous. Crucially at the end of each experiment the dialysis tubing can be discarded as a new one is relatively cheap (≈£60 for a box of 40).

The rest of the flow circuit consisted of gas-permeable silicone tubing with an internal diameter of 6mm and an external diameter of 10mm (Portexsil, Scientific Laboratory Supplies Ltd., Wilford Industrial Estate, Nottingham), 0.5 litre water-jacketed reservoir bottle (Radnoti Glass Technology Inc., Monrovia, CA, USA) connected to a water bath at 37°C, bioreactors and various 2-way connectors. The reservoir bottle has 3 input ports and one outflow port. One input port was for the medium returning from the flow circuit. One input port is designed for bubbling of gas into the medium collected within the bottle and one was open to the air outside. The latter two were covered with a sterile 22μm filter to allow gas exchange without entry of infectious materials. This was mainly to equalise pressures between the flow circuit and the outer atmosphere. The flow circuit was assembled in a sterile hood after autoclaving all the parts. The bioreactors and much of the gas-permeable tubing was designed to remain within a standard 37°C/5%CO_2 incubator to ensure optimum growth conditions. This meant that two lengths of tubing had to be connected to the rest of the flow circuit after being fed through side holes in the incubator. This potential source of infection was overcome by covering the distal part of the tubing with

autoclaved foil and only removing it once the tubing was back in the flow hood, thus ensuring that the inside and end of the tubing had remained sterile.

The final design is shown in Figure 7.5 complete with monitoring equipment for flow rate and pressure measurements. This consisted of a Transonic Medical Flowmeter system, and a Millar Mikro-tip catheter transducer for pressure. The circuit was circulated with perfusion solution. Shear stress at the wall of the graft and its compliance could be monitored with a Doppler ultrasound.

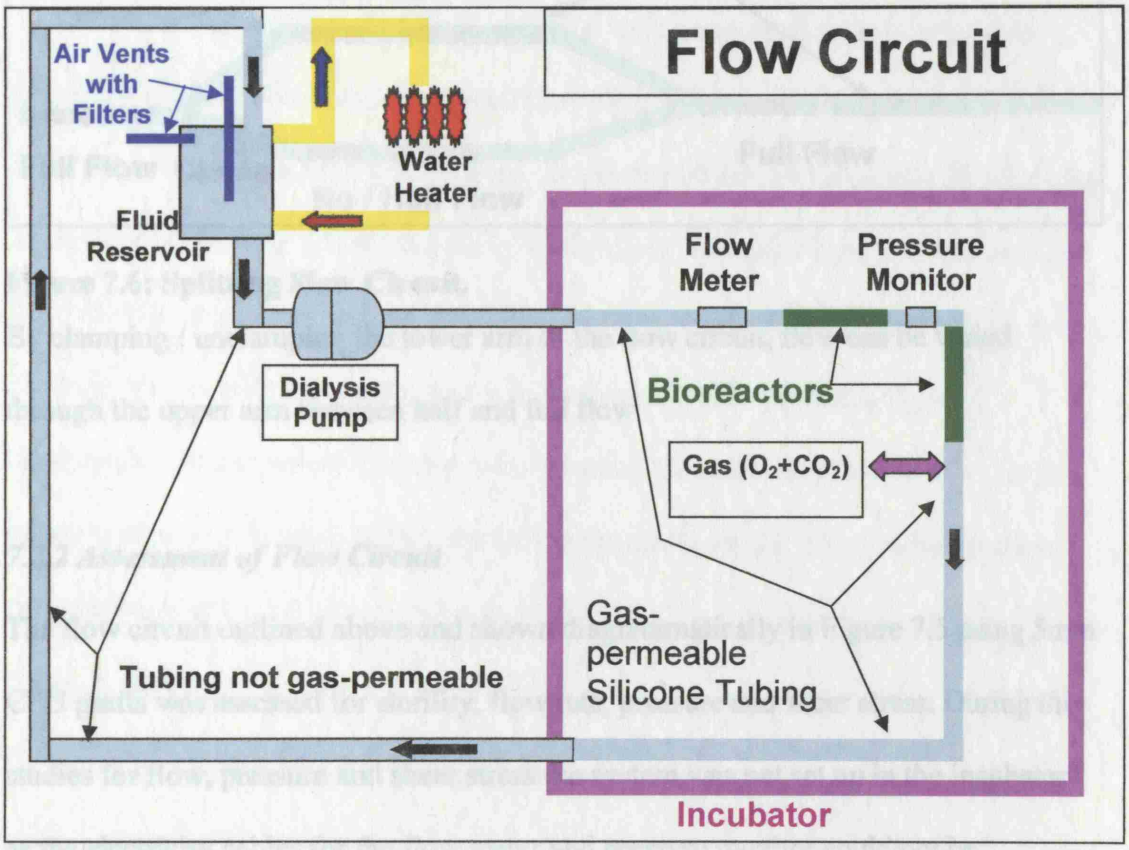


Figure 7.5: Flow Circuit

The flow rate through an individual length of tubing can however be further reduced using Y-shaped connectors to divide flow between 2 arms. This was done to vary flow – and therefore also shear stress – between different bioreactors during the same

experiment as shown in Figure 7.6. Therefore half the bioreactors could experience full flow whilst the other half only had half flow. By clamping the arm without any bioreactors, all the bioreactors could experience full flow.

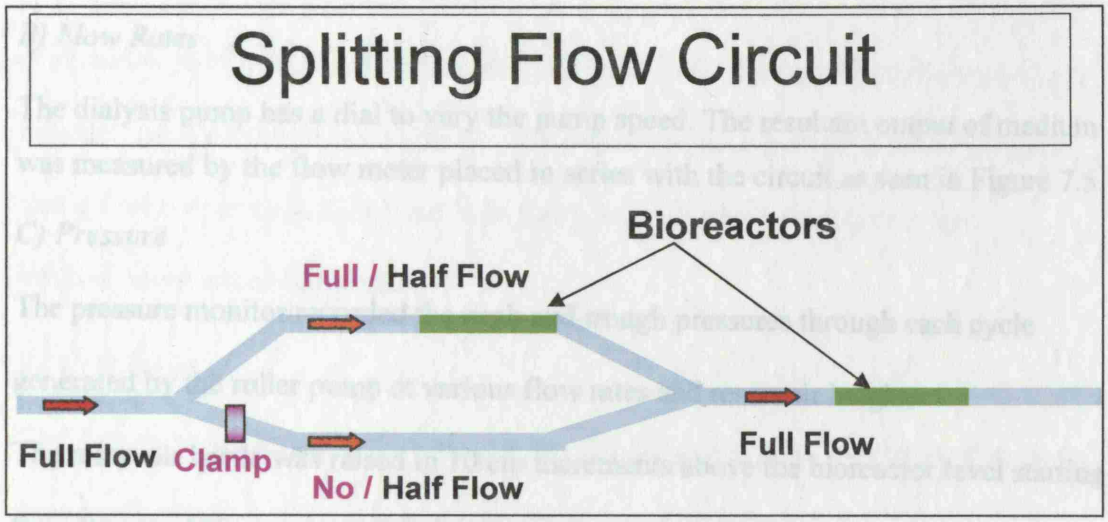


Figure 7.6: Splitting Flow Circuit.
By clamping / unclamping the lower arm of the flow circuit, flow can be varied through the upper arm between half and full flow.

7.2.2 Assessment of Flow Circuit

The flow circuit outlined above and shown diagrammatically in Figure 7.5 using 5mm CPU grafts was assessed for sterility, flow rate, pressure and shear stress. During the studies for flow, pressure and shear stress the system was not set up in the incubator as the electricity cables for the flow meter and pressure monitor could not be accommodated into the incubator. Furthermore, the grafts were not lined with cells as the flow meter and pressure monitor could not be adequately sterilised.

A) Sterility

Each time the flow circuit was constructed, the medium was assessed for sterility initially by simply observing the colour of the medium and then microscopic analysis of the medium using a phase contrast microscope.

B) Flow Rates

The dialysis pump has a dial to vary the pump speed. The resultant output of medium was measured by the flow meter placed in series with the circuit as seen in Figure 7.5.

C) Pressure

The pressure monitor recorded the peak and trough pressures through each cycle generated by the roller pump at various flow rates and reservoir heights.

The reservoir bottle was raised in 10 cm increments above the bioreactor level starting from 0 cm to 100 cm. At each height level flow was increased from 0 to 250 ml/min in 25 ml/min increments except for the fact that the lowest stable flow the pump could produce was 35 ml/min. Furthermore the number of pulses produced by the compression of the peristaltic pump was recorded manually at each flow rate. Each pulse equates to a 'cardiac cycle', thus allowing one to derive a 'pulse rate' in beats per minute (bpm).

D) Shear Stress

Calculations of shear stress required the use of an ultrasound device with a wall tracking system to determine graft radius. This was performed by Professor Alexander Seifalian, who simply supplied the results of these studies to me: as such they have not been included in the results section. See appendix 6 for more details.

7.3 Results

A) Sterility

The flow circuit was constructed 12 times and run for periods running from one day to several months (median = 2 weeks).

It remained sterile on 11 occasions, the only time it did not remain sterile was due to incorrect construction because the air filters between the reservoir bottle and the air outside were 45μm not 22μm and therefore unable to protect the circuit from infiltration by micro-organisms.

B) Flow Rates

The flow meter tallied with the flow dial on the dialysis pump. The speed of medium through the dialysis tubing ranged from 35 to over 500 ml/min.

The modification of Figure 7.6 was able to divide flow rates through either arm of the split circuit in two.

C) Pressure

Table 7.1 shows the impact of various flow rates on the frequency generation of pulses or beats. As can be seen from Figure 7.7 the relationship between flow and pulse rate is linear with one extra beat generated for every 4.2ml increase in flow rate.

Table 7.2 shows the recorded pressures at the different flow rates and reservoir heights. The pressure monitor noted the peak and trough pressures of the pressure wave developed by the pump, which equates to the systolic peak and diastolic trough found in the arterial blood pressure wave.

Flow Rate: ml/min	0	35	50	75	100	125	150	175	200	225	250
Pulse Rate: bpm	0	8	12	18	24	30	36	42	48	54	60

Table 7.1: Pulse Rate at Various Flow Rates.

Key: ml/min = millilitres per minute; bpm = beats per minute

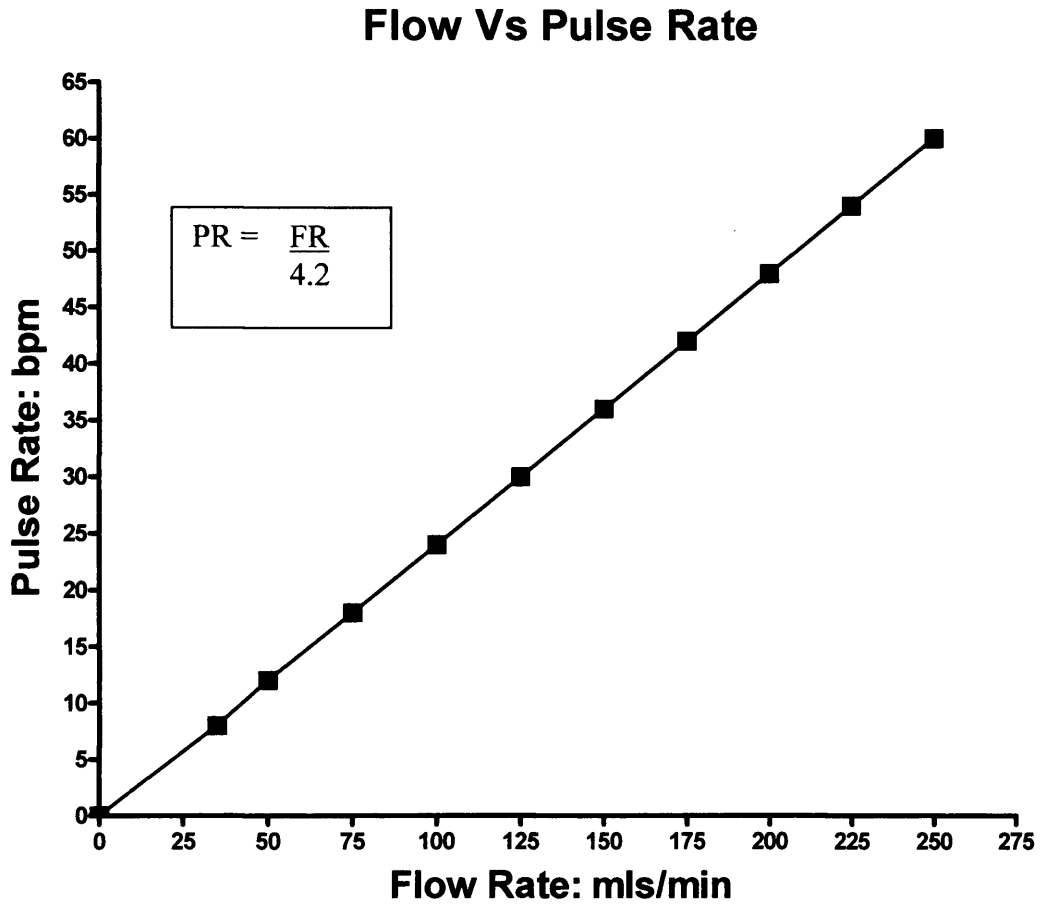


Figure 7.7: Pulse Rate at Various Flow Rates.

Increasing pulse rate with increasing flow rate.

Key: mls/min = millilitres per minute; bpm = beats per minute; PR= pulse rate (bpm); FR = flow rate (mls/min)

Pressure mmHg	Reservoir Height: cm										
	0	10	20	30	40	50	60	70	80	90	100
Flow Rate: ml/min 0	18/18 (18)	25/25 (25)	31/31 (31)	36/36 (36)	42/42 (42)	50/50 (50)	57/57 (57)	63/63 (63)	70/70 (70)	76/76 (76)	82/82 (82)
35	23/16 (20)	31/27 (29)	36/30 (32)	40/35 (37)	48/42 (44)	53/48 (50)	60/55 (57)	66/62 (64)	75/69 (71)	80/74 (76)	85/79 (82)
50	24/16 (20)	32/27 (29)	36/30 (32)	41/35 (37)	48/42 (44)	54/48 (50)	61/55 (57)	67/62 (64)	75/69 (71)	80/74 (76)	85/79 (82)
75	28/13 (20(	35/28 (30)	37/31 (33)	42/35 (37)	49/43 (45)	55/48 (51)	62/55 (58)	68/62 (64)	76/69 (71)	81/73 (77)	86/80 (82)
100	33/10 (22)	37/19 (30)	41/19 (33)	53/16 (37)	56/30 (43)	64/33 (51)	64/43 (56)	72/50 (64)	78/50 (70)	92/55 (77)	95/62 (82)
125	39/6 (25)	43/14 (31)	47/17 (34)	60/15 (38)	67/22 (45)	74/28 (52)	81/34 (58)	89/40 (65)	95/47 (72)	100/53 (77)	105/60 (82)
150	49/8 (27)	54/12 (32)	56/12 (35)	64/12 (38)	72/21 (45)	80/24 (52)	87/31 (59)	93/39 (65)	99/47 (72)	105/51 (77)	111/57 (83)
175	59/7 (28)	64/11 (33)	66/11 (36)	66/12 (39)	76/19 (46)	81/25 (52)	89/30 (59)	97/37 (66)	104/44 (73)	110/49 (78)	115/56 (84)
200	63/5 (29)	69/9 (33)	72/8 (36)	71/12 (39)	79/19 (47)	85/25 (53)	93/31 (60)	101/36 (66)	108/43 (73)	115/48 (79)	123/56 (84)
225	66/2 (29)	71/4 (32)	76/7 (36)	80/9 (40)	90/16 (47)	97/22 (53)	108/28 (60)	110/35 (67)	115/43 (74)	126/47 (79)	134/53 (84)
250	68/1 (29)	73/2 (30)	80/5 (36)	90/6 (40)	97/4 (48)	105/19 (54)	114/26 (61)	120/33 (68)	127/40 (74)	131/45 (80)	139/49 (85)

Table 7.2: Impact of Flow Rate and Fluid Height on Pressure.

Key: Pressure described as peak/trough (mean) – equivalent to systolic/diastolic (mean); ml/min = millilitres per minute; mmHg = millimetres of Mercury; cm = centimetres.

Figure 7.8 demonstrates how increasing flow of medium causes - irrespective of reservoir height - the systolic pressure to rise, diastolic pressure to fall and mean pressure to increase slightly.

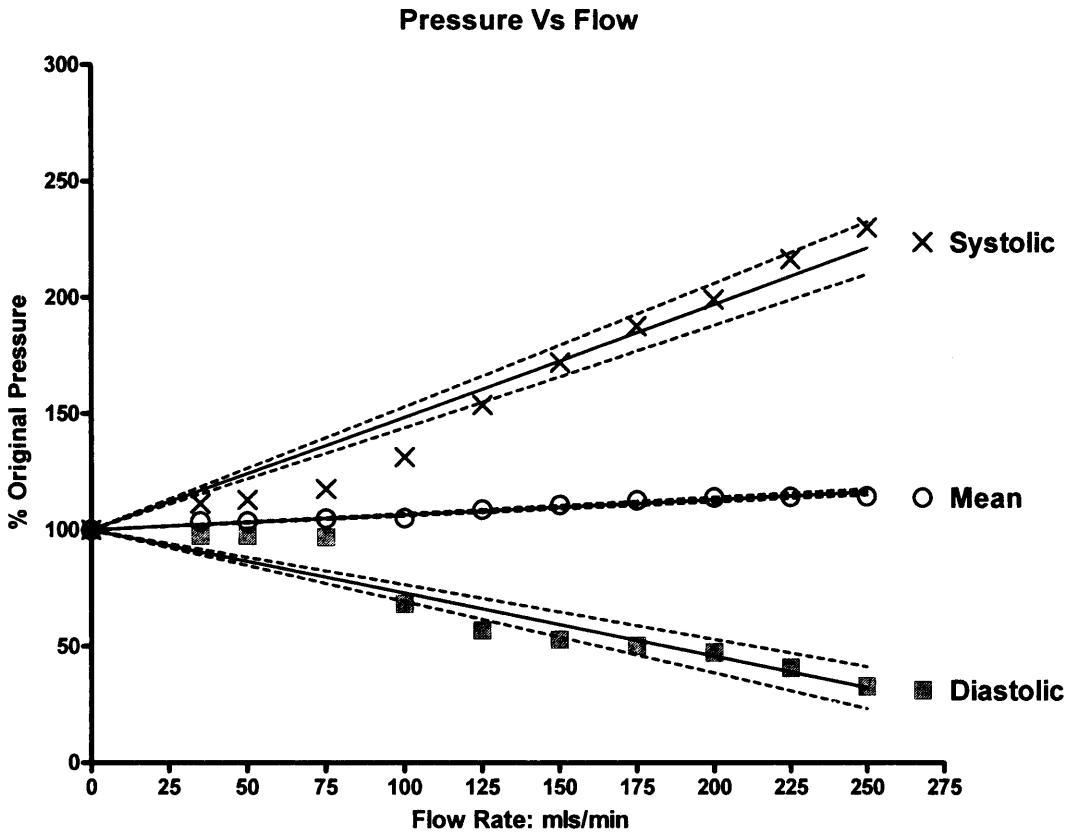


Figure 7.8: Pressure Change with Increasing Various Flow Rates.
With increasing flow rates, systolic pressure rises, diastolic pressure falls and mean pressure increases slightly.

Key: mls/min = millilitres per minute; full lines represent predicted values as determined by linear regression whereas dashed lines represent the calculated 95% confidence intervals.

Figure 7.9 shows that with increasing height, pressure rises linearly: more so for systolic than diastolic pressures.

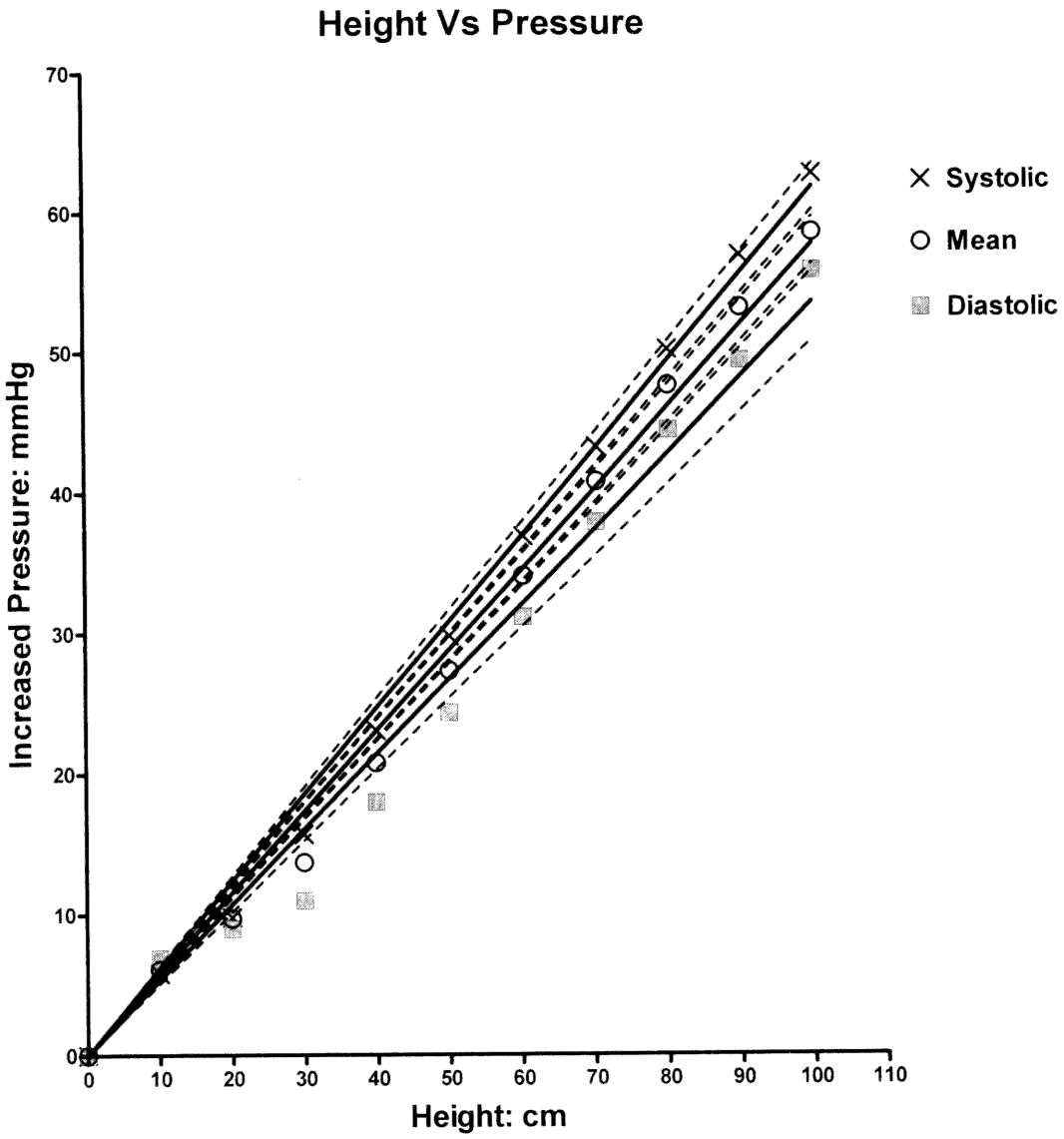


Figure 7.9: Pressure Change with Increasing Height of Reservoir

With increasing height, pressure rises linearly: more so for systolic than diastolic pressure.

Key: mmHg = millimetres of Mercury; cm = centimetres; full line = predicted values by linear regression; dashed lines = calculated 95% confidence intervals.

7.4 Conclusion

The bioreactor design and flow circuit developed here meets the four key requirements of a successful design as espoused by Bancroft(317).

1. Deliver flow through the scaffold and not around it.
    a. The bioreactor and flow circuit design ensured all flow was through the scaffold lumen.
2. Repeatable, consistent and controllable flow rate
    a. Flow rates between 35-500 mls/min were readily and reliably achieved.
    b. Flow rates were controlled by the pump speed and the flow rate dial on the pump was confirmed to be accurate using an independent flow meter.
3. Sterilisable and able to remain sterile
    a. The bioreactor parts are entirely autoclavable and sterility did not prove a problem, except when the Perspex barrels developed cracks after several autoclave cycles. Furthermore the design allowed medium exchange using a sterile technique.
    b. The flow circuit retained sterility whenever it was correctly set up.
4. Reasonable to operate: not too complex.
    a. The bioreactor is simple to construct from its parts and the flow circuit simply involves connecting together lengths of sterile tubing.
    b. The parts are readily available for the flow circuit from commercial suppliers. Although the bioreactor itself was custom made it is a relatively simple design made from standard and inexpensive materials.

c. The costs of the design are mainly one-off: a roller pump (in our case a disused dialysis pump), a standard laboratory incubator (37°C & 5%CO_2) and a half-litre water-jacketed reservoir bottle.

Experiments on the flow circuit revealed that the pressure in the circuit increases linearly with increasing reservoir height. Increasing the flow rate of the medium increases the pulse rate, systolic and mean pressure, whereas the diastolic pressure decreases so that the pulse pressure (systolic-diastolic) rises.

7.5 Summary

This chapter has demonstrated the development of an *in vitro* flow circuit that is cheap to set up and reproduce, easy to maintain, sterile in the long-term and reliably replicates the flow rates and pressures experienced in the human body. The bioreactors can be used for a host of tissue engineering applications and allow sequential seeding of cells, static culture and then dynamic culture under shear stress within the flow circuit. Indeed the bioreactor and flow circuit described could be used for all the potential uses expected of flow circuits: investigating the impact of mechanical forces, culturing cells into tissues and finally assessment of tissues themselves.

The impact of these variables, especially with respect to cell retention under the physiological flow conditions of human arteries, is the subject of the next chapter.

Chapter 8

ROLE OF PRE-CONDITIONING IN OPTIMISING CELL RETENTION TO COMPLIANT POLY(CARBONATE-UREA)URETHANE SCAFFOLDS

8.1 Introduction

This chapter examines the impact of 'pre-conditioning' on the retention of initially SMCs and then ECs (on a pre-lined SMC layer) onto the CPU graft, once it has been exposed to physiological flow rates and pressures.

'Pre-conditioning' has been shown to positively influence the development of tissue-engineered grafts. Here the vascular construct is exposed, *in vitro*, to pulsatile flow and pressure. This has been shown to enhance cell proliferation, tissue formation and mechanical properties(188;310;316). Our laboratory has previously shown that pre-conditioning with physiological shear stress using a flow circuit developed in-house can significantly enhance EC retention, viability and morphology. ECs seeded onto polymer grafts have specific problems with regard to cell attachment and retention(111). A coating of SMCs has been reported to enhance EC retention under physiological shear stress(330;331).

The aim of this study was to examine the effect of pre-conditioning on a tissue-engineered vascular construct consisting initially of scaffold and SMCs alone; and then ECs in our scaffold-SMC-EC hybrid graft exposed to physiological shear forces.

8.2 Methods and Materials

The validated flow system of Giudiceandrea et al(329) was modified to simulate *in vitro* the pulsatility and flow waveform including reverse flow, pressures, and degree of oxygenation and pH of physiological femoral artery circulation *in vivo*. This model was used to accurately determine SMC retention on grafts exposed to physiological shear stress. The model was set up as described in section 7.5.

8.2.1 SMC: Static vs. Constant Pre-conditioning

To investigate the impact of pre-conditioning, SMCs were radiolabelled as per the method outlined in appendix 2. The grafts were then lined with radiolabelled SMCs as per appendix 3, except that the grafts were not cut along their length, but cut into 5cm lengths with both ends plugged, rotated continuously for one hour in an in-house designed rotator before being incubated with medium in tissue culture flasks for one day in static culture. Then, half of the grafts were left in static culture with regular medium exchanges, whilst the other half were pre-conditioned at low shear stress (1-2 dynes/cm^2) in the flow circuit(118) as shown in Figure 7.5. In both experiments the grafts were incubated at 37°C and 5% CO_2. After a week all the grafts were put into the flow circuit under pulsatile physiological shear stress levels (25 dyne/cm^2), with dynamic scintigraphy images of the grafts acquired using a gamma camera(118;279;329).

Radioactivity from the ^{111}In-oxine-labelled SMCs was measured using a gamma camera scanner linked to an image processing system. The section of the circuit containing the vascular grafts was positioned over the gamma camera and imaged throughout the perfusion period. An on-line workstation recorded all images within 64 x 64 matrices. The initial 6 images were each acquired over 5 minutes, with 22 subsequent images acquired over a 15 minute span and used to generate time-activity

curves corrected for background, spontaneous indium leakage, and isotope decay (half-life of ^{111}In=68 hours). Cell attachment (*CA*) with respect to time was calculated from the equation below, where $(t)_n$ is time in minutes, $(t)_0$ is immediately before initiation of flow, and *CPMG* and *CPMB* are the counts per minute over the graft and background computed from analysis of dynamic scintigraphy images. Grafts were perfused for eight hours.

$$CA = \frac{Cells(t)_n}{Cells(t)_0} \times 100 = \frac{CPMG(t)_n - CPMB(t)_n}{CPMG(t)_n} \times 100$$

After the flow circuit data was collected, segments of grafts were sent for analysis using scanning electron microscopy.

8.2.2 EC: Static vs. Constant Pre-conditioning

To investigate the impact of pre-conditioning on retention of ECs, grafts were lined with ^{111}Indium-radiolabelled ECs as per the method outlined in appendix 2. After this – as per the SMCs - the grafts were rotated for a period of one hour and then left for a day in static culture to allow cell attachment. Half the grafts were left in static culture whilst the other half were pre-conditioned in the flow circuit described in section 8.2.1. In both experiments the grafts were incubated at 37°C and 5% CO_2. After 2 hours, all the grafts were put into a pulsatile flow circuit and dynamic scintigraphy images were acquired using a gamma camera(118;279;329) as per the method in section 8.2.1.

After the flow circuit data was collected, segments of grafts were sent for analysis using scanning electron microscopy. Some graft segments were stained using the standard Haematoxylin and Eosin (H&E) stain as follows:

1. Grafts segments fixed in acetone for 10 minutes.
2. Segments then air dried.
3. Wash in distilled H_2O.
4. Stain in Harris's Haematoxylin for 5 minutes.
5. Wash in tap water.
6. Dip slides in 0.5% acid alcohol for 1-2 seconds.
7. Wash in tap water.
8. Blue in Scots solution for a few seconds.
9. Wash in tap water.
10. Stain in Eosin for 15 seconds and wash in tap water.
11. Dehydrate rapidly in graded alcohols 70%, 95%, 100%.
12. Clear in xylene.
13. Mount in DPX and add cover slip.

In order to visualise the stained grafts a very powerful light was used to shine through the thickness of the graft in order to see the stained cells using a light microscope.

8.2.3 EC: Constant vs. Incremental Pre-conditioning

The above experiment (8.2.2) was repeated but this time comparing constant but low pre-conditioning over a week with incremental pre-conditioning whereby after 3 days the shear stress was increased by increasing the flow. To do this the flow circuit was set up with the modification enabling splitting of flow shown in Figure 7.6.

For the first 3 days the arm without bioreactors was clamped to ensure identical flow throughout all bioreactors. Then after 3 days the clamp was removed and the flow doubled. Therefore those bioreactors in the split part of the circuit received constant flow and shear stress (1-2 dyne/cm^2) but those bioreactors positioned after the two arms of the split in the circuit had re-united now had doubled flow and shear stress.

After a total of one week the circuit was again set up with arterial flows, pressure and shear stress (25 dyne/cm^2), with cell retention measured dynamically on a gamma camera.

After the gamma camera experiment, the grafts were sent for analysis of cell viability using Alamar Blue assay as per Appendix 4 and nucleic acid levels using the Pico Green Assay as per Appendix 5. Some samples were also sent for SEM visualisation of the surface.

Data Analysis and Statistical Methods

Data are presented with mean $\pm$ standard deviation (SD). For each of the experiments, the initial values of absolute radioactive levels between the two groups of pre-conditioned grafts were compared using the two-tailed T-test. For the time-activity-retention data, at each time point the two groups were compared using the Mann-Whitney test. Furthermore the two curves were then compared using a 2-way ANOVA. The Alamar Blue and Pico Green assay data were compared using the two-tailed T-test. The number of grafts in each group was 6 except for the incremental versus static pre-conditioning experiment where the numbers were 5 per group.

8.3 Results

8.3.1 SMC: Static vs. Constant Pre-conditioning

Before exposure of the cells to the shear force of arterial pulsatile blood flow, there was 8% lower cell count as determined by radioactivity levels on the vascular graft in the preconditioned group compared to the static group, but this difference was not statistically significant (303±22 vs. 280±35, p=0.106 [t-test]).

Upon commencement of the pulsatile flow circuit, the percentage of original radioactivity was significantly lower in the static grafts (81.3±2.7 vs. 93.2±1.8 for

pre-conditioned grafts) after only 5 minutes ($p<0.0022$ [Mann-Whitney]). When assessing absolute values, however, the difference only became significant after 60 minutes ($p=0.0087$ [Mann-Whitney]) because of the higher initial values in the static group (Figure 8.1). At the end of the study period of 8 hours, the values had plateaued, with the final % values being a mean of 56.7 ± 7.0 for the static group and 76.2 ± 6.5 for the pre-conditioned group ($p=0.0022$ [Mann-Whitney]). The two curves were significantly different ($p= 0.0005$ [2-way ANOVA])

Figure 8.2 shows SEM images of the two groups of grafts, with clearly greater number of cells on the pre-conditioned grafts.

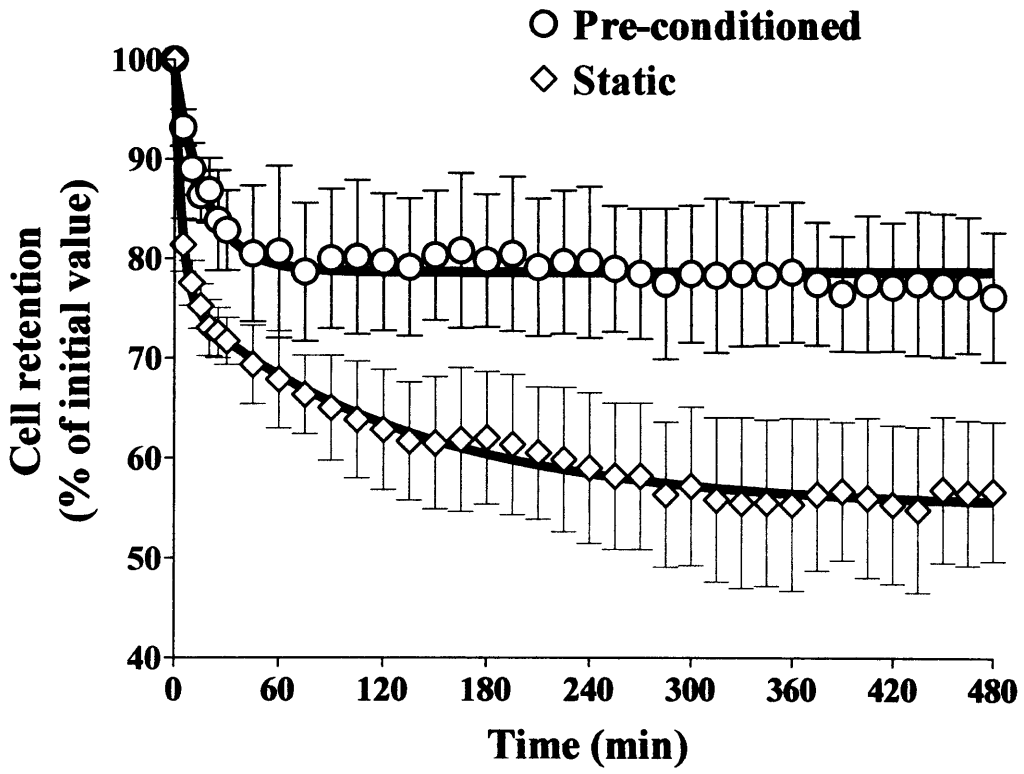


Figure 8.1: Effect of Pre-conditioning on Smooth Muscle Cell Retention to CPU

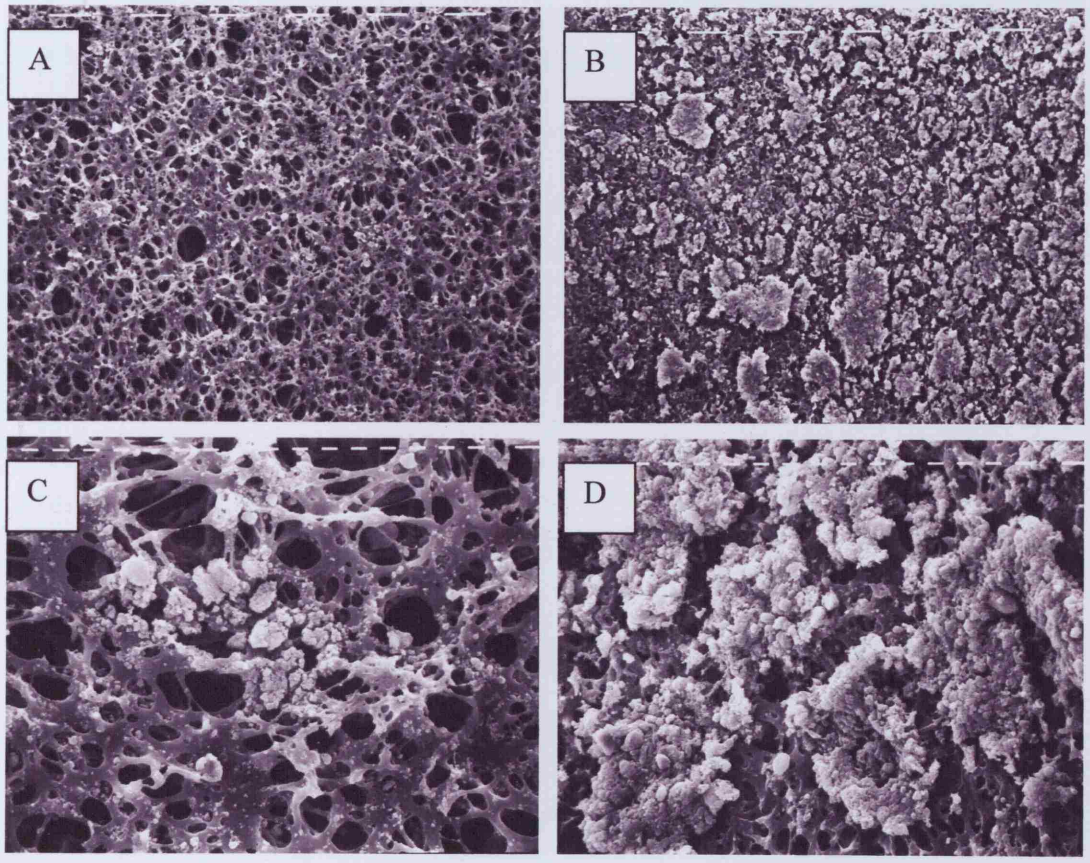
All values are mean $\pm$ standard deviation: n=6. Pre-conditioned grafts had significantly higher levels of cell retention.

Figure 8.2: Scanning Electron Micrographs of Smooth Muscle Cells on CPU Grafts

LHS: Static CPU: (A) top: *x60.5*; (C) bottom: *x242 magnification*

RHS: Pre-conditioned CPU: (B) top: *x60.5*; (D) bottom: *x242 magnification*

More cells are visible on the pre-conditioned grafts.



8.3.2 EC: Static vs. Constant Pre-conditioning

Before exposure of the cells to the shear force of arterial pulsatile blood flow, there was 5% lower cell count as determined by radioactivity levels on the vascular graft in the preconditioned group compared to the static group, but this difference was not statistically significant (12164±4451 vs. 11536±1710, p=0.7536 [t-test]).

Figure 8.3 shows that upon commencement of the pulsatile flow circuit, the percentage of original radioactivity was significantly lower in the static grafts (82.2±3.1 vs. 91.0±3.8 for pre-conditioned grafts) after 150 minutes (p=0.0286 [Mann-Whitney]). At the end of the study period of 8 hours, the values had barely begun to plateau for both the static and pre-conditioned groups. However there was a significant difference between the final % values with a mean of 45.6±2.3 for the static group and 67.4±4.0 for the pre-conditioned group (p=0.0286 [Mann-Whitney]). The two curves were significantly different (p= 0.0014 [2-way ANOVA]).

Figure 8.4 shows SEM images of the two groups of grafts and images under light microscopy after the grafts had been stained with haematoxylin and eosin – the dark spots representing cell nuclei.

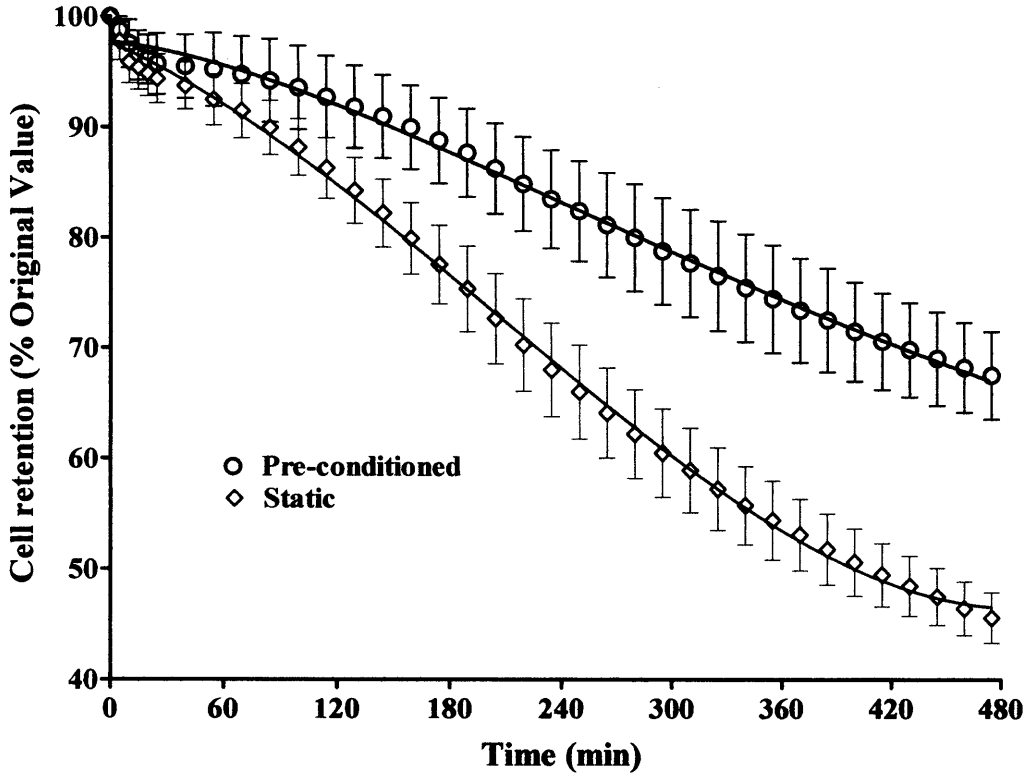


Figure 8.3: Effect of Pre-conditioning on Endothelial Cell Retention to CPU.

All values are mean ± standard deviation: n=6. Pre-conditioned grafts had significantly higher levels of cell retention.

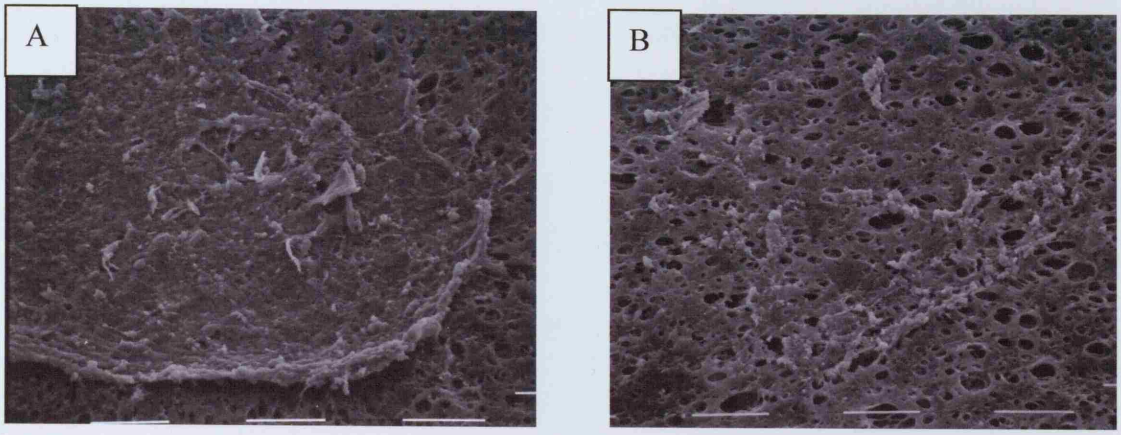


Figure 8.4: Smooth Muscle Cell –Endothelial Cell CPU Grafts.

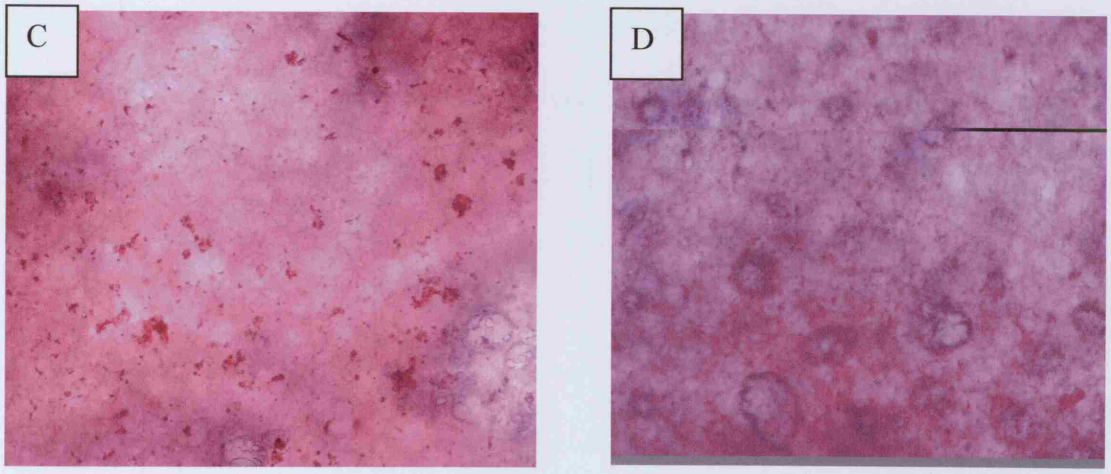
Above: Scanning Electron Micrographs (x220 magnification).

(A) Pre-conditioned CPU; (B) Static CPU.

Below: Grafts stained with Haematoxylin & Eosin (x200 magnification).

(C) Pre-conditioned CPU; (D) Static CPU.

More cells are visible on the pre-conditioned grafts suggesting greater cell attachment on pre-conditioned grafts.



8.3.3 *Constant vs. Incremental Pre-conditioning*

Before exposure of the cells to the shear force of arterial pulsatile blood flow, there was 3% lower cell count as determined by radioactivity levels on the vascular grafts in the constant preconditioned group compared to the incremental group, but this difference was not statistically significant (172±39 vs. 177±11, p=0.7968 [t-test]).Figure 8.5 shows that after commencement of the pulsatile flow circuit, the percentage of original radioactivity was significantly lower in the constantly pre-conditioned grafts (64.2±7.7 vs. 76.8±1.5 for incrementally pre-conditioned grafts) after 255 minutes (p=0.0159 [Mann-Whitney]). At the end of the study period of 8 hours, the values had begun to plateau for both groups. However there was a significant difference between the final % values with a mean of 56.8±8.9 for the constant group and 71.8±2.7 for the incrementally pre-conditioned group (p=0.0159 [Mann-Whitney]). The two curves were significantly different (p= 0.0117 [2-way ANOVA])

Figure 8.6 shows a SEM image of the cells on an incrementally pre-conditioned graft. After the grafts were exposed to arterial pulsatile flow, the cells on both the incrementally and constantly pre-conditioned grafts showed equal levels of viability - as shown in the Alamar Blue Assay (Figure 8.7(a) – p = 0.8063) - and nucleic acid – as shown in the Pico Green Assay (Figure 8.7(b) – p = 0.1479).

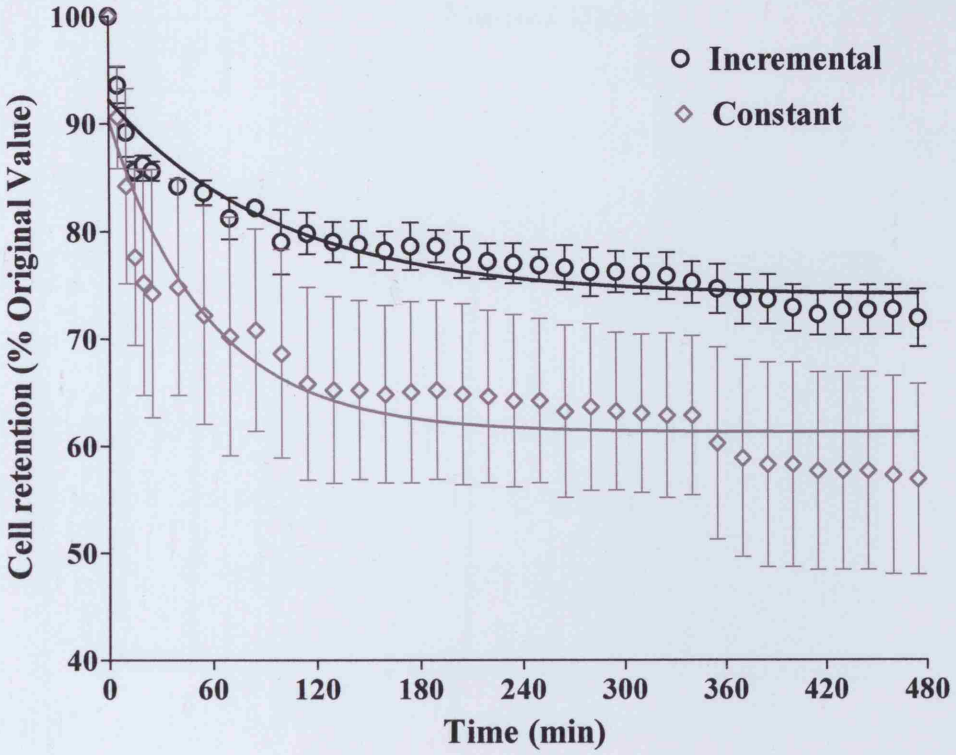


Figure 8.5: Effect of Variations in Pre-conditioning on Endothelial Cell Retention to CPU

All values are mean ± standard deviation: n=5. Incrementally pre-conditioned grafts had significantly higher levels of cell retention than constantly pre-conditioned grafts.

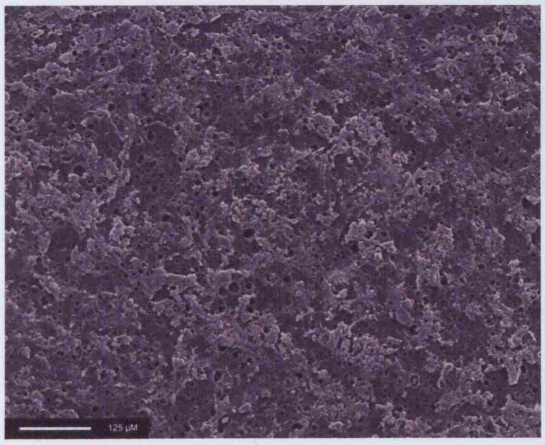


Figure 8.6: Scanning Electron Micrograph of Smooth Muscle Cell–Endothelial Cell CPU Graft.

Incrementally Pre-conditioned CPU: *x160 magnification.*

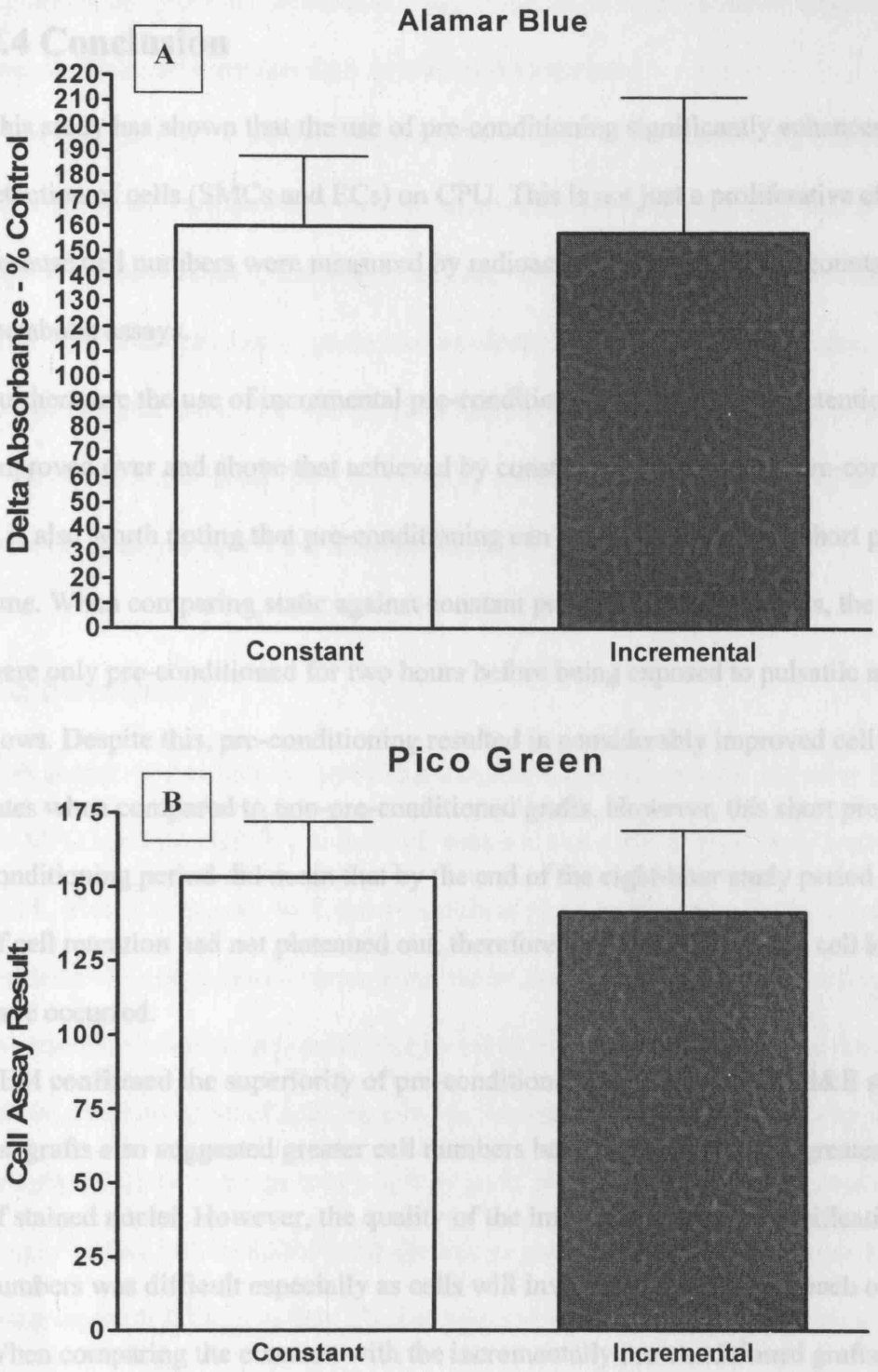


Figure 8.7: Effect of Variations in Pre-conditioning on Cellular: (a) Viability; (b) DNA

No significant difference between constant and incremental pre-conditioning.

Key: Values are mean ± standard deviation

8.4 Conclusion

This study has shown that the use of pre-conditioning significantly enhances the retention of cells (SMCs and ECs) on CPU. This is not just a proliferative effect because cell numbers were measured by radioactivity rather than cell counts or metabolic assays.

Furthermore the use of incremental pre-conditioning shows that cell retention can be improved over and above that achieved by constant low shear-stress pre-conditioning. It is also worth noting that pre-conditioning can work with relatively short periods of time. When comparing static against constant pre-conditioning for ECs, the grafts were only pre-conditioned for two hours before being exposed to pulsatile arterial flows. Despite this, pre-conditioning resulted in considerably improved cell retention rates when compared to non-pre-conditioned grafts. However, this short pre-conditioning period did mean that by the end of the eight-hour study period the level of cell retention had not plateaued out, therefore with more time more cell loss would have occurred.

SEM confirmed the superiority of pre-conditioned over static grafts. H&E staining of the grafts also suggested greater cell numbers based upon the visibly greater number of stained nuclei. However, the quality of the images meant that quantification of cell numbers was difficult especially as cells will invariably be overlying each other.

When comparing the constant with the incrementally pre-conditioned grafts, both had reached a plateau by the end of the eight-hour observation period, with a significantly higher level for the incremental regime. The reason for reaching a plateau level in this experiment but not in the previous experiment using static pre-conditioning was probably the time for pre-conditioning was one week in this experiment, compared to

2 hours in the previous experiment. This is not surprising as cells do require some time to settle and establish firm attachments to surfaces.

There was no significant difference between the levels of viable cells and nucleic acid detected on the incrementally and constantly pre-conditioned grafts. Whilst at first glance this may seem to contradict the data from the gamma camera, one must remember that both sets of grafts had an identically pre-conditioned underlayer of smooth muscle cells. The Alamar Blue and Pico Green assays would therefore have included both SMCs and ECs in the assay results whereas the gamma camera would have focussed purely on the radiolabelled ECs.

8.5 Summary

This chapter shows that the use of pre-conditioning enhances the retention of SMCs to the CPU scaffold and ECs to the CPU scaffold even after a SMC layer has been laid down. This is true even with short periods of pre-conditioning of only 2 hours. Furthermore a regimen of incremental rather than constant pre-conditioning enhances the retention of cells. Increasing the period of pre-conditioning reduces the time before a plateau level of cells remains on the grafts. This has important implications in optimising the cell layers of a hybrid graft. Importantly it means that cells can not simply be laid into a scaffold and allowed to grow simply in static culture before being implanted into a patient. Rather a period of time for pre-conditioning needs to be incorporated into the developing graft.

Further work now needs to be done to optimise the level of shear stress, its duration and the protocol involved.

Chapter 9

SUMMARY OF THESIS AND GENERAL DISCUSSION

This thesis has investigated the possibility of developing a hybrid cardiovascular bypass graft for small diameter arterial bypass based on a scaffold of compliant CPU, cells and extracellular matrix.

Chapter 1 described the need for novel small diameter compliant bypass grafts because of the low patency of currently available prosthetic graft such as PTFE and Dacron. This poor patency of prosthetic grafts can partly be explained by compliance mismatch between elastic artery and the stiffer graft leading to intimal hyperplasia. The Biomaterials and Tissue Engineering Centre of the Royal Free and University College Medical School have developed a small diameter compliant bypass graft for lower limb bypass (CPU). This graft has been manufactured to have compliance similar to human arteries and has previously been shown - both *in vitro* and *in vivo* - to be biostable. CPU is now commercially available for haemodialysis and is in a pilot study for peripheral vascular bypass - above knee femoral-popliteal bypasses in patients without suitable vein and otherwise thought to have very poor chances of success with prosthetic graft (personal communication). The CPU graft's superiority to current prosthetic grafts in terms of improved compliance and ability to attach and retain cells makes it a good biological scaffold material for use in developing a tissue-engineered blood vessel.

Chapter 2 reviews the various tissue engineering approaches to producing alternatives for small diameter arterial bypass. These approaches can be summarised according to the approach to the key components of the conduit: whether a mandrel or an actual scaffold was used; was the scaffold natural or synthetic - whether the synthetic

scaffold is biodegradable or not. Finally, were cells or extracellular matrix added or not.

Chapter 3 represents the initial stage of hybrid graft development by investigating sources for human vascular smooth muscle cells. For experimental purposes only two reliable sources were found – human saphenous vein from varicose vein surgery and human umbilical cord blood vessels. Extracting SMCs was successfully achieved using a combined digestion and explantation method developed in-house. Immunohistochemistry confirmed the cells were SMCs as they stained positively for alpha-actin but negatively for von Willebrand factor (EC marker) and fibroblasts. The immunohistochemical characterisation was used for four separate SMC populations. Whilst it could be argued that this should have been done for every extraction, the characteristics of SMCs are apparent in the hill-and-valley growth pattern visible under the light microscope every time SMC extraction and growth was done. Furthermore the reagents for immunohistochemistry are prohibitively expensive for routine use. The extraction success rate of 60% for saphenous vein and 50% for umbilical cord is far from ideal but the failures were mainly due to technical errors whilst on the 'learning curve'. Furthermore the vessels were not harvested primarily with a view to cell extraction and so the care of handling to preserve the vessel was not optimal. In contrast a dedicated procedure to harvest vessel solely for the purposes of cell extraction could be expected to have a much higher success rate. This would appear to be the case with much of the literature - which does not really quote success rates for SMC extraction from human vessels - but usually refers to procedures in animals where vessel harvest was for cell extraction.

Unsurprisingly extracted neonatal cord SMCs were found to be faster growing than adult SMCs. This would agree with the work of others who have extrapolated declining SMC proliferation rates with increasing donor age to conclude that by 105 years human SMCs would not proliferate at all(332). Similarly population doublings, *in vitro* lifespan and protein synthesis decline with rising age of cell donor(333-335). This is an important clinical consideration when postulating time scales for *in vitro* culture of cells for eventual seeding onto the graft scaffold. Both types of SMCs could return to a contractile state after serial passaging and Vitamin C offered no advantages in terms of detectable matrix production.

An interesting consideration is whether SMCs are overall beneficial at all. As mentioned in section 1.3, one of the principle reasons for graft failure is neointimal hyperplasia due to proliferation of vascular SMCs and ECM synthesis(63;64). Therefore adding SMCs could be considered to be detrimental and be associated with an increased risk of graft failure. Indeed the success of two-stage seeding onto prosthetic grafts(119;120) suggests that one does need a SMC layer. However, SMCs are known to interact with ECs and support their development on a prosthetic scaffold(330;331). Furthermore, as mentioned in section 1.3, the source of these SMCs is debateable and may not be from the arterial media(72-76) but from the stem or progenitor population circulating in the blood but originating from the marrow(77-80). Therefore avoiding a SMC layer may not abolish the risk of neointimal hyperplasia.

A hybrid graft requires ECs even more than SMCs. Ideally from a clinical standpoint a single rather than a separate source of both cells would reduce the trauma inflicted on a patient. Chapter 4 showed how a sequential enzymatic digestion method allows

extraction of both ECs and SMCs from the same vessel. This worked reliably (67%) for umbilical cords using the cannulation protocol, the failures again due to technical errors and perhaps delay from cord harvest to enzymatic digestion. The main reason for delay was that requests for umbilical cord to the Labour ward were made the night before collection was possible - leading to a delay of up to 16 hours. Given the ease and success of the cannulation technique, there seemed little purpose in pursuing the more demanding methodologies of eversion and scraping.

Extraction of ECs from saphenous vein proved unsuccessful by any methodology. Cannulation failed for technical reasons – it proved impossible to create a watertight seal for the enzyme solution to remain intralumenal and hence digest the EC layer over the requisite 10-15 minutes. Invariably the solution would leak out of holes in the side. Even when the vessel was pre-tested with sterile saline and all leaks repaired with 5/0 prolene, after a few minutes of intralumenal enzymatic digestion the leaks would either re-appear or new ones would reveal themselves. Surprisingly, the eversion and scraping methods proved fruitless also in extracting ECs from stripped adult saphenous vein. This conflicts with reports in the literature of successful extraction of ECs from saphenous vein, but I believe this is because the method of extraction of saphenous vein was not stripping but avulsion or dissection(262). Unfortunately no such source was available and the only saphenous veins received had been extracted using the stripping technique which denudes the luminal surface of its EC layer as was confirmed by SEM pictures of the lumen of freshly stripped varicose vein. In principle therefore a method for extraction of both SMCs and ECs from the same vessel has been established. However, it is true to say its feasibility with adult rather than neonatal vessels remains unproven at this stage, though

theoretically a dedicated and careful harvesting procedure of adult vein as used for bypass grafting should result in a successful extraction of both SMCs and ECs.

Tissue engineering depends on the successful adherence and proliferation of cells to extracellular material. The hybrid graft requires a layer of SMCs to be laid on the surface of the compliant graft scaffold. This cell attachment needed to be investigated and optimised. In this and other regards, ECM molecules have a critical impact on cells – conveying mechanical and chemical stimuli, influencing cellular shape, actin cytoskeleton organisation and transcription activity. These effects seem to be mediated by 'signals' embedded in the form of short amino acid sequences within ECM molecules. Certain peptides belong to a group of surface modifiers critical to enhancing cell interactions with biomaterials, including improved cell adhesion. Cell adhesion comprises four partially overlapping stages: initial cell attachment (the tethering of a cell to a substratum), cell spreading, organisation of actin cytoskeleton and formation of focal adhesions(266).

The interaction between cells and ECM through integrins and other cell surface adhesion receptors, is important not just for SMC adhesion but also migration, proliferation, contraction, differentiation and apoptosis(268). Integrins are important in SMC adhesion to ECM proteins like collagen(336). Vitronectin and fibronectin are ECM proteins that mediate attachment of SMCs to both natural scaffolds like collagen and synthetic scaffolds like polyesters of lactic and glycolic acid(337). RGD is a tripeptide sequence (Arginine-Glycine-Aspartate) found in extracellular matrix proteins like fibronectin. It is the binding motif for cell surface integrin receptors and has been investigated extensively(111;230-233). Our laboratory has shown that RGD

when covalently bonded to CPU and particularly when this was in association with heparin, significantly enhanced the retention and viability of seeded ECs(141).

The experiments in chapter 5 looked at the ways of optimising cell adhesion to a biomaterial. Initially the use of attachment factors was investigated. These included ECM proteins like collagen and FN. Given FN's prominent role in cell adhesion, a variant known as superfibronectin was also used. The remaining factors were peptides based on RGD which as mentioned is critical to cell adhesion. In particular I looked at repeating sequences of RGD in FEPP and its more positively-charged counterpart FEPP+. I found that although all attachment factors except fibronectin had a higher mean proportion of SMCs adhering to CPU, the only one to do this to statistical significance was FEPP+. The explanation for the superiority of FEPP+ would be its repeating sequences of RGD, which would enhance the binding of SMCs principally through integrin receptors to the CPU surface coated with RGD peptides and its positive charge which could attract the negatively-charged surface of cells.

The use of peptides on the surface of biomaterials does have its disadvantages. Firstly, these proteins must be isolated from organisms and purified, and so they may prove to be antigenic and potentially an infection risk. Furthermore they may undergo proteolytic degradation(267). Another concern is that they may impair cell viability, mobility, matrix production and proliferation(230;233;338). It must be stated that for vSMCs reduced mobility, matrix production and proliferation is desirable in the long-term, though in the short-term getting a confluent cell layer to establish upon a scaffold is crucial for hybrid graft development. Although these experiments were unable to determine if the attachment factors affected cell proliferation, the Alamar blue data excluded any detrimental effect on cell viability, with all attachment factors except for FN showing statistically significant higher viability values.

A final problem with the use of attachment factors refers to the way in which they are presented to the biomaterial and then the cells themselves. Ideally peptides should be linked to the biomaterial in a stable manner to ensure that they can withstand the contractile forces of cells so that focal adhesions are formed. Also unattached peptides can be internalised by the cells or block cell adhesion receptors which otherwise could be utilised for attachment to the scaffold surface(267).

Hersel(267) undertook a detailed review of the options for stable binding of peptides to scaffolds. Briefly the two main methods are derivitisation of polymers for RGD introduction and immobilising RGD peptides on the polymer surface. Derivitisation may involve introducing functional groups like hydroxyl-, amino-, or carboxyl groups. Methods for this include blending with other polymers containing functional groups, co-polymerisation of peptides with scaffold material, using interpenetrating networks of other materials, chemical treatment or physical methods like track-etching.(267)

Immobilising RGD peptides onto the polymer surface can sometimes be achieved using a simple coating procedure where the peptide has for example been endowed with a sticky tail. However, in most cases RGD peptides are linked to polymers via a stable covalent amide bond. Methods include use of a peptide coupling agent and chemoselective ligation using selected pairs of functional groups to form stable bonds without a coupling agent(267).

The Biomaterials and Tissue Engineering Centre (BTEC) at the Royal Free & University College Medical School has used patented spacer arm technology to chemically bond peptides to the surface of the CPU graft. RGD and heparin enhanced the adhesion of ECs to the CPU compared to controls consisting of native graft, and graft with preclot, fibronectin, gelatin and collagen(284). Furthermore the RGD and

heparin combination enhanced cell retention when exposed to pulsatile arterial flow(141).

In chapter 5 the peptides were simply coated onto the surface and so it could rightly be argued that perhaps the results do not accurately reflect the potential impact of the various peptides had they been tethered to the CPU surface in a more rigorous way. A possible solution to the question of how much of the peptides actually bound to the surface of the CPU would be to analyse the CPU-peptide surface. Methods for this include water contact analysis, ATR-FTIR (attenuated total reflection - Fourier transform infra-red spectroscopy), FTIR itself if samples can be made as thin films, X-ray photoelectron spectroscopy (XPS), amino-acid analysis, radioassays, assays and ELISAs. Detection of the microdistribution of RGD peptides has not been solved yet but possible methods include the use of high-resolution XPS and AFM (Atomic Force Microscopy) using antibody-coated AFM tips(267).

Whilst cell adhesion to RGD-peptide coated polymers is known to be time-dependent with cell spreading noted to be occurring as late as 80 hours(339), leaving the cells for too long a period before assessment of adhesion allows for cell proliferation to adulterate the results. It is therefore difficult to decide what time point to select when assessing cell adhesion. Most reports use between 1-4 hours. Removing the non-adherent cells can be done by washing, shaking or centrifuging(340;341). Determining the cells attached can be done by quantifying the cells removed, colorimetric methods(342;343), DNA fluorescence(344), radioisotope assays(280;345;346) or manual counting.

The assessment of cell adhesion to CPU was done using a radioisotope assay. This mitigates against the effect of cell proliferation as cell division has no impact upon the overall level of radioactivity. However, an obvious criticism is that radioactivity is a

surrogate marker of cells and can always leach out. The degree of this leakage has been shown by some to be minimal (223;347) and by others to be quite significant(280), especially over longer periods of time(348). Accordingly, it was suggested that the difference may partly be related to the cell source and type and so any study using ^{111}Indium-oxine requires leakage studies to be performed. However, as agreed by Stansby et al, our studies were looking more at qualitative differences rather than absolute numbers of cells present(280). Therefore our conclusions on the relative amount of cells present with different attachment factors remain valid. This is true in later chapters where different pre-conditioning regimens are compared as the cells used in every arm of the experiment are identical. Furthermore, supporting evidence for relative cell numbers was garnered from cell viability studies using the redox reagent Alamar Blue.

I left the cells to adhere over a 48 hour period initially. As mentioned, some reports suggest a longer period of time (80 hours)(339) would have allowed for possibly more adherence, this has to be counterbalanced by the need for medium exchange over such a long period. This in itself could have disrupted the adhesion process. Furthermore, other reports suggest that cells can adhere to scaffolds, with appropriate attachment factors like FN, within time periods of as little as five minutes and resist quite high detachment forces(349). The experiments in chapter 5 showed that there was no difference in adherence whether cells were left for a 24 or 48-hour period.

Increasing cell concentration resulted in increasing adherence up to values of half a million cells per square centimetre. Given that a confluent tissue culture flask of 75cm^2 gives between 1-4 million cells, this level of cell concentration would seem excessive. However, the CPU is a porous graft and so it is quite conceivable that cells penetrate through the graft's 'holes'. Furthermore SEM pictures clearly show cells

aggregate in islands together and are almost never seen in isolation. As mentioned, loss of cell attachment results in apoptosis in anchorage-dependent cells(350) like SMCs. It therefore appears that seemingly supraconfluent levels of cells are needed to ensure sufficient cell survival on CPU, based on adequate cell-cell as well as cell-scaffold contact. This is similar to the need for 'sodding' of ECs for successful seeding of prosthetic grafts(119;120). What has not been studied but may be of critical importance is the role of the CPU's honeycomb structure. For SMCs it has been recognised that higher scaffold void fractions (increased surface area to volume ratios) improve cell adhesion and ECM production especially with increased pore size(351). Furthermore the scaffold itself may determine cell shape - through the nature of the relationship between the polymer and the pores within it - which in itself can determine cell survival over and above the degree of attachment through integrin binding(273). Such a detailed look at the physical architecture of CPU remains to be performed, but would rely upon a high degree of consistency in architecture which again has not been verified by the manufacturer.

For the hybrid graft to be feasible in the long-term it is imperative that cells are able to actually remain and grow on CPU. Chapter 6 shows quantitatively over time cell proliferation using assays of cell viability (Alamar Blue) and nucleic acid levels (Pico Green). Whilst the levels of increase were not spectacular, this is partly a reflection of the high seeding densities required to 'coat' the scaffold surface for successful attachment to occur as described previously in chapter 5. Furthermore a qualitative method to visualise the SMCs on the CPU scaffold using retroviral gene transduction to insert GFP into the SMCs was described. Fluorescent microscopy was then able to confirm visually that the SMCs do actually grow on the CPU graft. The one major

drawback with GFP is that not all cells are successfully transduced and so the images gained are not a complete representation of the cells on the surface of the graft. It is technically possible to select out only the GFP-containing cells, using for example magnetic dynabeads containing the antibody to GFP, however, this would be an expensive step and involve another application to the Genetics Modification Committee. Ideally a technique to visualise the same cells growing and developing on the graft over a longer period of time is required, but issues of sterility proved problematic as the fluorescent microscope needs to be in very close proximity to the cells. Perhaps modern confocal microscopes can overcome this difficulty.

In contrast, more conventional methods, using for example fluorescent carbocyanine dyes(352), result in the immunofluorescent dyes being passed onto daughter cells in equal amounts causing progressive dilution with each cell division. In addition there are concerns regarding the toxicity of such dyes(288). The use of GFP overcomes these problems.

Furthermore, transduction of GFP would allow GFP-labelled cells to be clearly distinguishable from host cells in an *in vivo* animal study, thus demonstrating whether cells in the graft were those transplanted or were replaced by host cells lacking GFP.

A successful hybrid graft requires a period of *in vitro* culture which allows development of the biological layers in a 3-dimensional environment, where ideally the cells and matrix are exposed to mechanical stimuli. Chapter 7 describes how a novel bioreactor design and innovative flow circuit was developed to allow long-term culture of the developing hybrid graft under optimal conditions of heat, nutrient supply and mechanical stimuli whilst maintaining sterility over periods of up to several months. This system allowed sequential seeding, culture and even testing of

tissues. The described flow circuit is cheap to assemble from readily available commercial parts with good reliability. Although the dialysis pump generated pressures and 'pulse rates' approximating to that found in the human body it lacks the dynamic changes that occur in the human body over seconds, minutes and even hours. A neat solution would perhaps be a computer algorithm to vary the pump output to at least mimic diurnal variation. This particular problem has not even really begun to be dealt with in the tissue engineering literature which remains fixed on static outputs. The testing described concentrated on mechanical indices (pressure, flow etc.) and we did not specifically test for biological indices like pH of the medium and oxygen levels. A collaboration with the Medical Physics department attempted to measure pH continuously but was found to be unreliable. However, sampling at regular intervals revealed that the flow circuit set-up – with a long length of gas permeable tubing within the 5%CO_2 / 37°C incubator – maintained a constant pH in the desirable range (7.35-7.45) and any movement from this could easily be detected by a change in the medium colour. Similarly, the incubator set-up allows adequate oxygen levels to be maintained by gas exchange across the gas-permeable tubing and through the reservoir bottle with its two gas-exchange ports. This is similar to growing cells in tissue culture flasks inside the incubator. Furthermore, experimental work in this department has shown adequate levels of oxygen to be maintained in the flow circuit over time(329).

The bioreactors themselves could be criticised as the Perspex barrels crack after several autoclaves but this would not be important in a clinical scenario where the bioreactor would almost certainly be single use to prevent cross-infection between patients.

Previous chapters have described how suitable cells have been isolated from human sources, attached to and then grown on the compliant graft scaffold. However, there is much work in the literature to show that cells simply wash off grafts when exposed to pulsatile flow and the high pressures of the arterial circulation. It is known though that mechanical stress orientates cells and the ECM both *in vitro* or *in vivo*(192-196;205-207;291;300-303;353;354). Pulsatile blood flow results in a mechanical stimulation composed of hydrostatic pressure, tangential shear stress and circumferential stretch-relaxation(291;292). Endothelial cells because of their contact with flowing blood or medium are exposed to all three forces(292). Furthermore exposure to shear stress has been shown to enhance endothelial cell attachment, retention and differentiation as well as being a critical factor in their biological regulatory function(326;355-358). Both shear stress and cyclic strain increase proliferation of endothelial cells, increase production of ECM molecules like collagen and fibronectin(292;293). SMC proliferation also is enhanced by shear stress(294).

Chapter 8 used these principles and the flow circuit developed in chapter 7 to 'pre-condition' CPU grafts lined with cells at pulsatile subarterial pressures and flows. Using ^{111}Indium radiolabelling of SMCs and following the radioactivity dynamically of grafts on a gamma camera, it was established that pre-conditioning significantly improves cellular retention once the cells on grafts are exposed to full arterial pressures and flow rates.

Thereafter ^{111}Indium radiolabelling was used again but this time with ECs to show that once again pre-conditioning enhances retention of cells once grafts are exposed to full arterial pressure and flow rates. This benefit can occur with as little as 2 hours of pre-conditioning, though this time period means that after 8 hours a baseline level of cell retention was not established, which suggests that pre-conditioning needs to be

done for longer in order to be truly effective in the long-term. This can be explained by the fact that cell attachment occurs in four overlapping stages, beginning with cell attachment which occurs within seconds to minutes. This allows cells to withstand gentle shear forces, without which cells could easily be rinsed off a surface. Then cells flatten as the cell membrane spreads to take its characteristic shape. Thirdly, actin is organised into microfilament bundles also known as stress fibres, Finally focal adhesions or contacts are formed, consisting of clustered integrins and other transmembrane, membrane-associated and cytosolic molecules which link ECM molecules to the cell's actin cytoskeleton(266).It is notable that previous studies have shown that a layer of SMCs itself enhances the retention of ECs(330;331).

Furthermore a regimen of incrementally increasing pre-conditioning and shear stress further enhances the retention of ECs when exposed to full arterial pressures and flow rates. This concurs with other studies in the literature showing that incremental pre-conditioning enhances retention of cells more than constant pre-conditioning at low shear stress(182). It seems that such a regimen is better at preparing the attached cells for the disruption caused by full arterial pressures and flows. By gently increasing the shear stress and cyclic strain cells can develop the ECM and adhesion molecules required to attach to each other and the scaffold surface without succumbing to the detachment forces of circulating medium.

Studies have also shown that exposure of tissue-engineered vessels to the mechanical stimulation of pulsatile flow increases mechanical strength of the constructs as measured by parameters like burst pressure(176;188;211;310;359;360). This is critical if implanted vessels are to avoid aneurysmal dilation and ultimately rupture once exposed to long-term arterial blood pressures. The optimum conditions for the pre-conditioning are still to be defined but some propose that the ideal for tissue-

engineered vessels may actually be fetal conditions(361), where the high pulse rate (165 beats per minute) has been shown to enhance collagen deposition and ECM turnover(324).

Future work

This thesis has gone through the sequential stages needed to develop a hybrid biological graft for arterial bypass consisting of a compliant scaffold, smooth muscle cells and endothelial cells. However, it would be correct to say that the various stages from cell isolation, scaffold-cell attachment, cell growth on the graft, extracellular matrix development and finally pre-conditioning to optimise cell retention need more and detailed work.

Firstly cell isolation needs really to be studied in the kind of patients who need arterial bypasses – elderly, often diabetic patients with renal impairment. The feasibility of cell isolation and growth will be very different from neonatal umbilical cords or even saphenous veins from relatively younger and healthier varicose vein patients. Furthermore, the reagents used for cell extraction and growth need to be those which are clinically approved for use in humans. Initial enquiries into such reagents could not be followed up for cost reasons as the safety requirements are understandably much more stringent than simple *in vitro* culture reagents.

An alternative emerging source for cells could be stem and progenitor cells. These cells can be isolated from bone marrow(152) or from the blood itself(147) using chemokines to stimulate release into the blood from the marrow of progenitor cells(149). These cells could then either be placed directly onto scaffold(182;201) or be initially transdifferentiated into either endothelial cells(362) or smooth muscle cells(363) and then used to form the biological layers of a hybrid graft.

The compliant graft scaffold needs an inner layer of SMCs and further work needs to be done to optimise the establishment of this SMC layer. This would involve determining the best initial seeding concentration of SMCs, the rotational velocity of initial seeding, together with the optimum settling time before exposure to pre-conditioning. Additional work on attachment factors and whether these should be bonded to the scaffold surface could also be rewarding.

Once the SMC layer has been optimised, similar experiments with endothelial cells need to be done – seeding density, rotational velocity and initial settling time prior to pre-conditioning. Further experiments looking at co-culture as opposed to staged culture of SMCs and then ECs could offer the possibility of a much simpler and possibly shorter period to completion of a layered hybrid graft.

With regard to pre-conditioning, its optimal duration, the level of shear stress and the best regimen of incremental shear stress have yet to be perfected. Finally perfusion of the outside of the hybrid graft with culture medium, versus the inside or even both sides should be looked at.

Another interesting study would have been to determine the thrombogenicity of the graft in its native and hybrid state. The difficulty being how to assess this. Our department has used instruments such as Thromboelastographs to assess new polymer formulations with reduced thrombogenic potential(364) but this is not feasible for scaffolds with cells lined on the surface. Potentially one could measure bleeding time on the surface of the finished graft, but the issue is complicated by potential immunologic considerations as the blood really should be from the same source as the SMCs and ECs.

A final consideration is that once all the above experiments have been optimised and the feasibility has been determined, what is the next step before offering this to patients? Traditionally this has been preceded by animal experiments: implanting either animal cells into the same animal or human cells into animals. However, invariably this methodology leads to false hope as animals for example readily endothelialise prosthetic grafts spontaneously. Indeed a recent review found a huge range of animals studied from mice and rats through rabbits and dogs to large animals like sheep and pigs and even primates. However, none of these animal models proved reliable for cardiovascular tissue engineering purposes (365). The scaffold itself obviously needs testing for biostability and toxicity purposes but for CPU this has already been done in a long-term dog model(133). Furthermore CPU is in clinical use as a haemodialysis graft, so safety really should not be a concern.

It is therefore worth omitting the animal experimentation stage to focus on clinical studies, initially offering it to those patients without suitable vein in areas where prosthetic bypasses are felt to be significantly inferior. This would include the coronary circulation and perhaps infra-inguinal bypass especially re-do surgery. The question always raised at this stage is that the time needed for cell culture makes it often inappropriate for such indications. One possible proposal is that vessel harvest could occur at an earlier stage in the disease's progression or for re-do surgery when the first operation is initially done. For example extra vein could be harvested, digested of cells which are then cultured up and frozen down and stored in liquid nitrogen. I have successfully used human cells that have been frozen for at least 2 years. This would cut the time, from request for use to implantation, down to just a few weeks rather than months. If hybrid vessels prove beneficial in such difficult clinical scenarios a full randomised control trial comparing it to prosthetic or even

venous grafting can then be initiated. Success here would prove an invaluable tool in the armamentarium of surgeons involved in arterial bypass surgery.

Appendices

Appendix 1 – Extraction of Smooth Muscle Cells

The following procedure was used to extract human SMCs:

1. Collect long saphenous vein or umbilical vein / artery specimen into sterile phosphate buffered saline (PBS) + 0.2% gentamicin (Sigma G-1397) solution.
2. Trim ends of vessel.
3. Cut to 15cm.
4. Strip adventitia.
5. Flush lumen with PBS + gentamicin solution.
6. Place in centrifuge tube with 7.5ml of sterile DMEM* (Sigma D6171) with 0.1% collagenase A (Boehringer Mannheim 103 586) + 0.05% elastase (Sigma E-6883).
7. Agitate for 45 minutes at 37°C.
8. Transfer vein into 7.5ml of fresh enzyme solution.
9. Agitate for 2 hours at 37°C.
10. Transfer vein into universal container with 20ml DMEM + 0.04% collagenase.
11. Agitate overnight for 16 hours at 37°C.
12. Remove remaining vein and place onto 25cc flask as an explant.
13. Carefully transfer solution to centrifuge leaving behind any visible vein particles.
14. Centrifuge at 140G for 7 minutes.
15. Pipette off supernatant.
16. Resuspend the cell plug in 1ml Smooth muscle cell (SMC) medium *.
17. Remove 40 µl for cell counting.
18. Add further 6ml SMC medium.
19. Transfer to 25cm^2 culture flask and incubate at 37°C in 5% CO_2 atmosphere.

SMC medium consisted of DMEM [Dulbecco's Modified Eagle's Medium] with HEPES modification, 10% FBS [Foetal Bovine Serum], 2mM L-Glutamine, 2.5 μg/ml Amphotericin B, 100 units/ml penicillin and 100 μg/ml streptomycin.

Appendix 2 – Radiolabelling Cells

To assess cell attachment to CPU, SMCs were radiolabelled using the following procedure:

1. Count cells to be radiolabelled.
2. Re-centrifuge to form cell pellet and pour off supernatant.
3. Re-suspend cells in 4ml of serum-free medium: Medium 199 or DMEM.
4. Add ^{111}Indium-Oxine at 1.8MBq/10^6 cells and incubate at 37°C for 15 minutes.
5. Re-centrifuge cell suspension at 300G for 7 minutes.
6. Pour off supernatant and re-suspend cells in 4 ml of serum-containing medium.
7. Incubate at 37°C for 5 minutes and repeat stages 5-7 twice.
8. Finally cells are ready to be diluted to an appropriate concentration, ready for seeding.

Appendix 3 – Coating of Grafts

1. Cut CPU Graft into appropriate lengths.
2. Plug one end and fill the whole graft with attachment factor. Now plug other end.
3. Place graft in sterile container and rotate on roller for a minimum of one hour.
4. After allowing sufficient time for graft surface to be coated, remove graft in sterile hood and cut along whole length and then into 1-2cm pieces.
5. Remove plunger from 2ml syringe and cut surface until horizontal.
6. Place cut graft piece with inner surface upwards onto plunger of 2ml syringe and push plunger back into syringe to 2ml mark.
7. Now add appropriate volume of radiolabelled cell solution through top of syringe and place sterile cap onto syringe.
8. After fixed period of time, aspirate with needle the solution in syringe. This is the aspirate.
9. Lavage 3 times with sterile PBS, collecting lavages each time – this is lavage sample.
10. Remove graft and place into sterile container with some SMC culture medium.
11. Take all grafts, aspirates, lavages and syringes and needles for counting radioactivity in gamma counter.
12. Cells on CPU graft segments can then have their viability determined with Alamar Blue redox assay – appendix 4.

Appendix 4 – Alamar Blue™ Viability Assay

1. Dilute Alamar Blue™ (Serotec Ltd., Kidlington, Oxford, UK) 1:10 with cell culture medium.
2. Add diluted Alamar Blue solution to cell-containing graft.
3. After specified time period, aspirate 50 or 100µl of Alamar Blue solution in contact with graft and place into well of 96-well plate.
4. Place 96-well plate into spectrophotometer and absorbance read spectroscopically at wavelengths of 570 and 630 nm (Labsystems Multiscan MS visible spectrophotometer).
5. Absorbance is compared to controls without any cells on at all.
6. Values can be taken at one fixed time point or over a time course with serial time points.

Appendix 5 – Pico Green® DNA quantitation Assay

1. Aspirate all medium on cell-containing surface followed by 3 lavages with sterile PBS.
2. Add 1ml of clear trypsin solution to the cell-containing surface to dislodge any cells.
3. Incubate at 37°Cfor 10 minutes with occasional gentle agitation.
4. Aspirate all trypsin solution and wash 3 times with sterile PBS collecting all washings in a 1.8ml PCR tube.
5. Aspirate and expel all collected trypsin and PBS washings through a 23G needle attached to a 2ml syringe 3 times.
6. Freeze at -4°C and then thaw a few days later.
7. Sonicate the PCR tubes for 5 minutes.
8. Mix contents thoroughly.
9. A set of standard controls consisting of serially diluted calf thymus DNA were prepared.
10. In 96-well plates: 100 µl aliquots of samples and controls were mixed with 100 µl 1:400 (v/v) dilution of Pico Green in 1x TE.
11. The solutions were then incubated for 5 minutes in the dark.
12. Measurements of emissions at 538nm after excitation at 485 nm were read in a Fluoroskan Ascent FL, Thermo Labsystems.

Appendix 6 – Calculation for Shear Stress

Shear stress itself is partly a reflection of the physical parameters of the flow circuit itself but also viscosity of the solution as defined by the Wormersley Equation(366):

$$\gamma = \frac{\alpha.\mu.U}{\sqrt{2}.R}$$

γ = Shear Stress; μ = viscosity; U = volumetric velocity; R = graft radius;

α = Wormersley parameter [$\alpha = R.\sqrt{\beta}/v$: β = angular velocity; v = kinematic velocity]

Publications (Peer-Reviewed)

Rashid ST, Fuller B, Hamilton G, Seifalian AM. DEVELOPMENT OF BIOLOGICAL GRAFTS. TISSUE ENGINEERING OF CONDUITS FOR BYPASS SURGERY: REVIEW.

Cell Proliferation 2004; 37(5): 351-66.

Rashid ST, Salacinski H, Button MJC, Fuller B, Hamilton G, Seifalian AM. CELLULAR ENGINEERING OF CONDUITS FOR CORONARY AND LOWER LIMB BYPASS SURGERY: ROLE OF CELL ATTACHMENT PEPTIDES & PRE-CONDITIONING IN OPTIMISING SMOOTH MUSCLE CELLS ADHERENCE TO POLYMER SCAFFOLDS: ORIGINAL ARTICLE.

European Journal of Vascular and Endovascular Surgery 2004; 27 (6): 608-16.

Rashid ST, Salacinski H, Hamilton G, Seifalian AM. THE USE OF ANIMAL MODELS IN DEVELOPING THE DISCIPLINE OF CARDIOVASCULAR TISSUE ENGINEERING: REVIEW.

Biomaterials 2004; 25 (9): 1627-37.

Seifalian AM, Tiwari A, Rashid ST, Salacinski H, Hamilton G. IMPREGNATION OF THE POLYMERIC GRAFT WITH ADHESIVES MOLECULES, TYPICALLY OLIGOPEPTIDES OR GLYCOPROTEIN IMPROVES RETENTION: LETTER

Artif Organs 2002; 26 (2): 209-10; discussion 210-1.

Published Abstracts

Rashid ST, Salacinski HJ, Button MJC, Tohill M, Fuller B, Seifalian A, Hamilton G. HYBRID BYPASS GRAFT MADE FROM COMPLIANT POLY(CARBONATE-UREA)URETHANE SCAFFOLD, HUMAN SMOOTH MUSCLE AND ENDOTHELIAL CELLS FROM THE SAME PATIENT. **British Journal of Surgery 2005; 92 (10): 1323**

Rashid ST, Salacinski H, Button MJC, Fuller B, Hamilton G, Seifalian AM. CELLULAR ENGINEERING OF BYPASS GRAFTS: ROLE OF CELL DENSITY, ATTACHMENT FACTORS AND PRECONDITIONING IN OPTIMIZING SMOOTH MUSCLE CELL ADHERENCE TO COMPLIANT POLY(CARBONATE–UREA)URETHANE SCAFFOLDS.

British Journal of Surgery 2004; 91: 1236.

Button MJC; Rashid ST, Fuller B, Hamilton G. THE BEHAVIOUR OF ADULT HUMAN SMOOTH MUSCLE CELLS IN A WHOLLY BIOLOGICAL TISSUE ENGINEERED ARTERIAL BYPASS GRAFT. **Eur Surg Res 2003; 35: 186.**

Rashid ST, Salacinski H, Button MJC, Fuller B, Hamilton G, Seifalian AM. CELLULAR ENGINEERING OF CONDUITS FOR CORONARY AND LOWER LIMB BYPASS SURGERY: ROLE OF CELL ATTACHMENT PEPTIDES & PRE-CONDITIONING IN OPTIMISING SMOOTH MUSCLE CELLS ADHERENCE TO POLYMER SCAFFOLDS.

Cell Proliferation 2003; 36: 238.

Rashid ST, Salacinski H, Fuller B, Hamilton G, Seifalian AM. ENGINEERING A GRAFT FOR CORONARY BYPASS SURGERY: ROLE OF CHEMICAL COATINGS TO ENHANCE ATTACHMENT OF SMOOTH MUSCLE CELLS TO COMPLIANT POLY(CARBONATE-UREA)URETHANE MATRICES.

European Cells and Materials 2002; 4; Supplement 2; 11-12.

Presentations – International Conferences

Rashid ST, Salacinski HJ, Button MJC, Fuller B, Hamilton G, Seifalian AM. A HYBRID BYPASS GRAFT – USING PRE-CONDITIONED SMOOTH MUSCLE CELLS & ENDOTHELIAL CELLS ON COMPLIANT POLY(CARBONATE-UREA)URETHANE SCAFFOLD. International Society for Applied Cardiovascular Biology 9th Biennial Meeting. Savannah, USA: **10-13 March 2004**.

Rashid ST, Salacinski HJ, Button MJC, Fuller B, Hamilton G, Seifalian AM. CELLULAR ENGINEERING OF CONDUITS FOR CORONARY AND LOWER LIMB BYPASS SURGERY: ROLE OF CELL ATTACHMENT PEPTIDES & PRE-CONDITIONING IN OPTIMISING SMOOTH MUSCLE CELLS (SMC) ADHERENCE TO POLYMER SCAFFOLDS. *European Society for Vascular Surgery XVII Annual Meeting: Dublin, Eire.* **5-7 September 2003.**

Button MJC; Rashid ST, Fuller B, Hamilton G. THE BEHAVIOUR OF ADULT HUMAN SMOOTH MUSCLE CELLS IN A WHOLLY BIOLOGICAL TISSUE ENGINEERED ARTERIAL BYPASS GRAFT. *European Society for Surgical Research. Ghent, Belgium.* **28-31 May 2003.**

Rashid ST, Salacinski H, Fuller B, Hamilton G, Seifalian AM. CELLULAR ENGINEERING OF CONDUITS FOR CORONARY AND LOWER LIMB BYPASS SURGERY: ROLE OF CELL ATTACHMENT PEPTIDES & PRE-CONDITIONING IN OPTIMISING SMOOTH MUSCLE CELLS (SMC) ADHERENCE TO POLYMER SCAFFOLDS. *International Meeting on TISSUE ENGINEERED BLOOD VESSELS 2003: Gothenburg, Sweden.* **26-7 April 2003.**

Presentations – National Conferences

Rashid ST, Salacinski HJ, Button MJC, Tohill M, Fuller B, Seifalian AM, Hamilton G. A HYBRID BYPASS GRAFT MADE FROM COMPLIANT POLY(CARBONATE-UREA)URETHANE SCAFFOLD, HUMAN SMOOTH MUSCLE AND ENDOTHELIAL CELLS FROM THE SAME PATIENT. *Society of Academic & Research Surgery Annual Forum: Newcastle, UK.* **12-4 January 2005.**

Rashid ST, Salacinski HJ, Button MJC, Fuller B, Hamilton G, Seifalian AM. CELLULAR ENGINEERING OF BYPASS GRAFTS: ROLE OF CELL DENSITY, ATTACHMENT FACTORS & PRE-CONDITIONING IN OPTIMISING SMOOTH MUSCLE CELL ADHERENCE TO COMPLIANT POLY(CARBONATE-UREA)URETHANE SCAFFOLDS. *Society of Academic & Research Surgery Annual Forum: Belfast, UK.* **14-6 January 2004.**

Rashid ST, Salacinski H, Button MJC, Fuller B, Hamilton G, Seifalian AM. CELLULAR ENGINEERING OF CONDUITS FOR CORONARY AND LOWER LIMB BYPASS SURGERY: ROLE OF CELL ATTACHMENT PEPTIDES & PRE-CONDITIONING IN OPTIMISING SMOOTH MUSCLE CELLS (SMC) ADHERENCE TO POLYMER SCAFFOLDS. *British Tissue Engineering Network (BRITE Net) Meeting for Cardiovascular Tissue Engineering - Stem Cells, Matrix Biology and Bioreaction. London, UK.* **10th July 2003.**

Rashid ST, Salacinski H, Fuller B, Hamilton G, Seifalian AM. ENGINEERING A GRAFT FOR CORONARY BYPASS SURGERY: ROLE OF CHEMICAL COATINGS TO ENHANCE ATTACHMENT OF SMOOTH MUSCLE CELLS TO COMPLIANT POLY(CARBONATE-UREA)URETHANE MATRICES. *Tissue and Cell Engineering Society (TCES) 2002: Glasgow, UK.* **19-20 September 2002.**

Prizes

The following prizes have been awarded in recognition of work that is in whole in part contained within this thesis.

2004 **Young Investigator Award for Outstanding Research**

International Society for Applied Cardiovascular Biology

2003 **Congress Travel Grant**

European Society for Vascular Surgery

2003 **Patey Prize** (co-author)

Society of Academic & Research Surgery Annual Forum

References

Reference List

(1) Dorland's Illustrated Medical Dictionary. 27 ed. Philadelphia: W.B. Saunders Co., 1988.

(2) Manson JE, Greenland P, LaCroix AZ, Stefanick ML, Mouton CP, Oberman A et al. Walking compared with vigorous exercise for the prevention of cardiovascular events in women. N Engl J Med 2002; 347(10):716-725.

(3) Jonason T, Bergstrom R. Cessation of smoking in patients with intermittent claudication. Effects on the risk of peripheral vascular complications, myocardial infarction and mortality. Acta Med Scand 1987; 221(3):253-260.

(4) Silagy C, Mant D, Fowler G, Lancaster T. Nicotine replacement therapy for smoking cessation. Cochrane Database Syst Rev 2000;(3):CD000146.

(5) Pickering TG. Lifestyle modification and blood pressure control: is the glass half full or half empty? JAMA 2003; 289(16):2131-2132.

(6) Appel LJ, Champagne CM, Harsha DW, Cooper LS, Obarzanek E, Elmer PJ et al. Effects of comprehensive lifestyle modification on blood pressure control: main results of the PREMIER clinical trial. JAMA 2003; 289(16):2083-2093.

(7) Effects of ramipril on cardiovascular and microvascular outcomes in people with diabetes mellitus: results of the HOPE study and MICRO-HOPE substudy. Heart Outcomes Prevention Evaluation Study Investigators. Lancet 2000; 355(9200):253-259.

(8) Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, Dagenais G. Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med 2000; 342(3):145-153.

(9) MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. Lancet 2002; 360(9326):7-22.

(10) Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998; 352(9131):837-853.

(11) Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. Lancet 1998; 352(9131):854-865.

(12) O'Grady H, Kelly C, Bouchier-Hayes D, Leahy A. Homocysteine and occlusive arterial disease. Br J Surg 2002; 89(7):838-844.

(13) Collaborative overview of randomised trials of antiplatelet therapy--I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. Antiplatelet Trialists' Collaboration. BMJ 1994; 308(6921):81-106.

(14) Stone GW. Primary angioplasty versus "earlier" thrombolysis--time for a wake-up call. Lancet 2002; 360(9336):814-816.

(15) Weaver WD, Simes RJ, Betriu A, Grines CL, Zijlstra F, Garcia E et al. Comparison of primary coronary angioplasty and intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review. JAMA 1997; 278(23):2093-2098.

(16) Malik I, Berger A. Coronary angioplasty and stenting. BMJ 2002; 325(7363):519.

(17) O'Neill WW, Grines CL. Surgery or stent? The gap continues to narrow. Lancet 2002; 360(9338):961-962.

(18) Detre K, Murphy ML, Hultgren H. Effect of coronary bypass surgery on longevity in high and low risk patients. Report from the V.A. Cooperative Coronary Surgery Study. Lancet 1977; 2(8051):1243-1245.

(19) Detre K, Peduzzi P, Murphy M, Hultgren H, Thomsen J, Oberman A et al. Effect of bypass surgery on survival in patients in low- and high-risk subgroups delineated by the use of simple clinical variables. Circulation 1981; 63(6):1329-1338.

(20) Long-term results of prospective randomised study of coronary artery bypass surgery in stable angina pectoris. European Coronary Surgery Study Group. Lancet 1982; 2(8309):1173-1180.

(21) Myocardial infarction and mortality in the coronary artery surgery study (CASS) randomized trial. N Engl J Med 1984; 310(12):750-758.

(22) Kumar P, Treasure T. Coronary artery bypass graft trials. Br J Hosp Med 1996; 56(1):33-36.

(23) Hadorn DC, Holmes AC. The New Zealand priority criteria project. Part 2: Coronary artery bypass graft surgery. BMJ 1997; 314(7074):135-138.

(24) Management of peripheral arterial disease (PAD). TransAtlantic Inter-Society Consensus (TASC). Eur J Vasc Endovasc Surg 2000; 19 Suppl A:Si-250.

(25) Loop FD, Lytle BW, Cosgrove DM, Stewart RW, Goormastic M, Williams GW et al. Influence of the internal-mammary-artery graft on 10-year survival and other cardiac events. N Engl J Med 1986; 314(1):1-6.

(26) Canver CC. Conduit options in coronary artery bypass surgery. Chest 1995; 108(4):1150-1155.

(27) Grondin CM, Campeau L, Lesperance J, Enjalbert M, Bourassa MG. Comparison of late changes in internal mammary artery and saphenous vein grafts in two consecutive series of patients 10 years after operation. Circulation 1984; 70(3 Pt 2):I208-I212.

(28) Barner HB, Sundt TM, III. Multiple arterial grafts and survival. Curr Opin Cardiol 1999; 14(6):501-505.

(29) Suma H. Arterial grafts in coronary bypass surgery. Ann Thorac Cardiovasc Surg 1999; 5(3):141-145.

(30) He GW. Arterial grafts for coronary artery bypass grafting: biological characteristics, functional classification, and clinical choice. Ann Thorac Surg 1999; 67(1):277-284.

(31) Barner HB. Arterial grafting: techniques and conduits. Ann Thorac Surg 1998; 66(5 Suppl):S2-S5.

(32) Cooper GJ, Underwood MJ, Deverall PB. Arterial and venous conduits for coronary artery bypass. A current review. Eur J Cardiothorac Surg 1996; 10(2):129-140.

(33) Kapadia NK, Kapadia S. The first ever radial artery as a femoro-popliteal bypass: a case report. Indian Heart J 1998; 50(4):474-475.

(34) Teodorescu VJ, Chun JK, Morrisey NJ, Faries PL, Hollier LH, Marin ML. Radial artery flow-through graft: A new conduit for limb salvage. J Vasc Surg 2003; 37(4):816-820.

(35) Harris PL, Veith FJ, Shanik GD, Nott D, Wengerter KR, Moore DJ. Prospective randomized comparison of in situ and reversed infrapopliteal vein grafts. [see comments]. British Journal of Surgery 1993; 80(2):173-176.

(36) Veith FJ, Moss CM, Sprayregen S, Montefusco C. Preoperative saphenous venography in arterial reconstructive surgery of the lower extremity. Surgery 1979; 85(3):253-256.

(37) Dardik H, Dardik II. Successful arterial substitution with modified human umbilical vein. Ann Surg 1976; 183(3):252-258.

(38) Kovalic AJ, Beattie DK, Davies AH. Outcome of ProCol, a bovine mesenteric vein graft, in infrainguinal reconstruction. Eur J Vasc Endovasc Surg 2002; 24(6):533-534.

(39) Martin RS, Edwards WH, Mulherin JL, Edwards WH, Jenkins JM, Hoff SJ. Cryopreserved saphenous vein allografts for below-knee lower extremity revascularization. Annals of Surgery 1994; 219(6):664-670.

(40) Hasson JE, Newton WD, Waltman AC, Fallon JT, Brewster DC, Darling RC et al. Mural degeneration in the glutaraldehyde-tanned umbilical vein graft: incidence and implications. J Vasc Surg 1986; 4(3):243-250.

(41) Snyder SO, Wheeler JR, Gregory RT, Gayle RG, Zirkle PK. Freshly harvested cadaveric venous homografts as arterial conduits in infected fields. Surgery 1987; 101(3):283-291.

(42) Callow AD. Arterial homografts. Eur J Vasc Endovasc Surg 1996; 12(3):272-281.

(43) Abbott WM. Prosthetic above-knee femoral-popliteal bypass: indications and choice of graft. Semin Vasc Surg 1997; 10(1):3-7.

(44) Johnson WC, Lee KK. Comparative evaluation of externally supported Dacron and polytetrafluoroethylene prosthetic bypasses for femorofemoral and axillofemoral arterial reconstructions. Veterans Affairs Cooperative Study #141. J Vasc Surg 1999; 30(6):1077-1083.

(45) Robinson BI, Fletcher JP, Tomlinson P, Allen RD, Hazelton SJ, Richardson AJ et al. A prospective randomized multicentre comparison of expanded polytetrafluoroethylene and gelatin-sealed knitted Dacron grafts for femoropopliteal bypass. Cardiovasc Surg 1999; 7(2):214-218.

(46) Pevec WC, Darling RC, L'Italien GJ, Abbott WM. Femoropopliteal reconstruction with knitted, nonvelour Dacron versus expanded polytetrafluoroethylene. J Vasc Surg 1992; 16(1):60-65.

(47) Lord RS, Nash PA, Raj BT, Stary DL, Graham AR, Hill DA et al. Prospective randomized trial of polytetrafluoroethylene and Dacron aortic prosthesis. I. Perioperative results. Ann Vasc Surg 1988; 2(3):248-254.

(48) Bergan JJ, Veith FJ, Bernhard VM, Yao JS, Flinn WR, Gupta SK et al. Randomization of autogenous vein and polytetrafluorethylene grafts in femoral-distal reconstruction. Surgery 1982; 92(6):921-930.

(49) Christenson JT, Broome A, Norgren L, Eklof B. Revascularization of popliteal and below-knee arteries with polytetrafluoroethylene. Surgery 1985; 97(2):141-149.

(50) Whittemore AD, Kent KC, Donaldson MC, Couch NP, Mannick JA. What is the proper role of polytetrafluoroethylene grafts in infrainguinal reconstruction? Journal of Vascular Surgery 1989; 10(3):299-305.

(51) Comparative evaluation of prosthetic, reversed, and in situ vein bypass grafts in distal popliteal and tibial-peroneal revascularization. Veterans Administration Cooperative Study Group 141. Archives of Surgery 1988; 123(4):434-438.

(52) Faries PL, LoGerfo FW, Arora S, Hook S, Pulling MC, Akbari CM et al. A comparative study of alternative conduits for lower extremity revascularization: all-autogenous conduit versus prosthetic grafts. Journal of Vascular Surgery 2000; 32(6):1080-1090.

(53) Faries PL, LoGerfo FW, Arora S, Pulling MC, Rohan DI, Akbari CM et al. Arm vein conduit is superior to composite prosthetic-autogenous grafts in lower extremity revascularization. Journal of Vascular Surgery 2000; 31(6):1119-1127.

(54) Pomposelli FB, Jr., Arora S, Gibbons GW, Frykberg R, Smakowski P, Campbell DR et al. Lower extremity arterial reconstruction in the very elderly: successful outcome preserves not only the limb but also residential status and ambulatory function. J Vasc Surg 1998; 28(2):215-225.

(55) Byrne J, Darling RC, III, Chang BB, Paty PS, Kreienberg PB, Lloyd WE et al. Infrainguinal arterial reconstruction for claudication: is it worth the risk? An analysis of 409 procedures. J Vasc Surg 1999; 29(2):259-267.

(56) Stonebridge PA, Prescott RJ, Ruckley CV. Randomized trial comparing infrainguinal polytetrafluoroethylene bypass grafting with and without vein interposition cuff at the distal anastomosis. The Joint Vascular Research Group. J Vasc Surg 1997; 26(4):543-550.

(57) Pappas PJ, Hobson RW, Meyers MG, Jamil Z, Lee BC, Silva MB, Jr. et al. Patency of infrainguinal polytetrafluoroethylene bypass grafts with distal interposition vein cuffs. Cardiovasc Surg 1998; 6(1):19-26.

(58) Miller JH, Foreman RK, Ferguson L, Faris I. Interposition vein cuff for anastomosis of prosthesis to small artery. Aust N Z J Surg 1984; 54(3):283-285.

(59) Taylor RS, McFarland RJ, Cox MI. An investigation into the causes of failure of PTFE grafts. Eur J Vasc Surg 1987; 1(5):335-343.

(60) Tyrrell MR, Wolfe JH. New prosthetic venous collar anastomotic technique: combining the best of other procedures. Br J Surg 1991; 78(8):1016-1017.

(61) Conte MS, Mann MJ, Simosa HF, Rhynhart KK, Mulligan RC. Genetic interventions for vein bypass graft disease: a review. J Vasc Surg 2002; 36(5):1040-1052.

(62) Virchow RR. Cellular Pathology. London: Churchill, 1860.

(63) Sottiurai VS, Yao JST, Flinn WR, Batson RC. Intimal hyperplasia and neo intima: an ultrastructural analysis of thrombosed grafts in humans. Surgery 1983; 93:809-817.

(64) Szilagyi DE, Elliott JP, Hageman JH, Smith RF, Dall'olmo CA. Biologic fate of autogenous vein implants as arterial substitutes: clinical, angiographic and histopathologic observations in femoro-popliteal operations for atherosclerosis. Ann Surg 1973; 178(3):232-246.

(65) Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362(6423):801-809.

(66) Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999; 340(2):115-126.

(67) Imparato AM, Bracco A, Kim GE, Zeff R. Intimal and neointimal fibrous proliferation causing failure of arterial reconstructions. Surgery 1972; 72(6):1007-1017.

(68) Gozna ER, Mason WF, Marble AE, Winter DA, Dolan FG. Necessity for elastic properties in synthetic arterial grafts. Can J Surg 1974; 17(3):176-179.

(69) Tiwari A, Cheng KS, Salacinski H, Hamilton G, Seifalian AM. Improving the patency of vascular bypass grafts: the role of suture materials and surgical techniques on reducing anastomotic compliance mismatch. Eur J Vasc Endovasc Surg 2003; 25(4):287-295.

(70) Bell PR. Interposition vein cuffs--are they effective? Cardiovasc Surg 1998; 6(1):17-18.

(71) Hillebrands JL, Klatter FA, Rozing J. Origin of vascular smooth muscle cells and the role of circulating stem cells in transplant arteriosclerosis. Arterioscler Thromb Vasc Biol 2003; 23(3):380-387.

(72) Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. Science 1973; 180(93):1332-1339.

(73) Hruban RH, Long PP, Perlman EJ, Hutchins GM, Baumgartner WA, Baughman KL et al. Fluorescence in situ hybridization for the Y-chromosome can be used to detect cells of recipient origin in allografted hearts following cardiac transplantation. Am J Pathol 1993; 142(4):975-980.

(74) Glaser R, Lu MM, Narula N, Epstein JA. Smooth muscle cells, but not myocytes, of host origin in transplanted human hearts. Circulation 2002; 106(1):17-19.

(75) Kennedy LJ, Jr., Weissman IL. Dual origin of intimal cells in cardiac-allograft arteriosclerosis. N Engl J Med 1971; 285(16):884-887.

(76) Dartsch PC, Bauriedel G, Schinko I, Weiss HD, Hofling B, Betz E. Cell constitution and characteristics of human atherosclerotic plaques selectively removed by percutaneous atherectomy. Atherosclerosis 1989; 80(2):149-157.

(77) Shimizu K, Sugiyama S, Aikawa M, Fukumoto Y, Rabkin E, Libby P et al. Host bone-marrow cells are a source of donor intimal smooth- muscle-like cells in murine aortic transplant arteriopathy. Nat Med 2001; 7(6):738-741.

(78) Saiura A, Sata M, Hirata Y, Nagai R, Makuuchi M. Circulating smooth muscle progenitor cells contribute to atherosclerosis. Nat Med 2001; 7(4):382-383.

(79) Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med 2002; 8(4):403-409.

(80) Grimm PC, Nickerson P, Jeffery J, Savani RC, Gough J, McKenna RM et al. Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal-allograft rejection. N Engl J Med 2001; 345(2):93-97.

(81) Begovac PC, Thomson RC, Fisher JL, Hughson A, Gallhagen A. Improvements in GORE-TEX(R) Vascular Graft Performance by Carmeda(R) BioActive Surface Heparin Immobilization. Eur J Vasc Endovasc Surg 2003; 25(5):432-437.

(82) Devine C, Hons B, McCollum C. Heparin-bonded Dacron or polytetrafluoroethylene for femoropopliteal bypass grafting: a multicenter trial. Journal of Vascular Surgery 2001; 33(3):533-539.

(83) Devine C, McCollum C. Heparin-bonded Dacron or polytetrafluorethylene for femoropopliteal bypass: five-year results of a prospective randomized multicenter clinical trial. J Vasc Surg 2004; 40(5):924-931.

(84) Tiwari A, Salacinski HJ, Hamilton G, Seifalian AM. Tissue engineering of vascular bypass grafts: role of endothelial cell extraction. Eur J Vasc Endovasc Surg 2001; 21(3):193-201.

(85) Zilla P, Siedler S, Fasol R, Sharefkin JB. Reduced reproductive capacity of freshly harvested endothelial cells in smokers: a possible shortcoming in the success of seeding? Journal of Vascular Surgery 1989; 10(2):143-148.

(86) Ortenwall P, Wadenvik H, Risberg B. Reduced platelet deposition on seeded versus unseeded segments of expanded polytetrafluoroethylene grafts: clinical observations after a 6-month follow-up. Journal of Vascular Surgery 1989; 10(4):374-380.

(87) Zilla P, Fasol R, Dudeck U, Siedler S, Preiss P, Fischlein T et al. In situ cannulation, microgrid follow-up and low-density plating provide first passage endothelial cell masscultures for in vitro lining. Journal of Vascular Surgery 1990; 12(2):180-189.

(88) Zilla P, Preiss P, Groscurth P, Rosemeier F, Deutsch M, Odell J et al. In vitro-lined endothelium: initial integrity and ultrastructural events. Surgery 1994; 116(3):524-534.

(89) Zilla P, Fasol R, Deutsch M, Fischlein T, Minar E, Hammerle A et al. Endothelial cell seeding of polytetrafluoroethylene vascular grafts in humans: a preliminary report. Journal of Vascular Surgery 1987; 6(6):535-541.

(90) Jensen N, Lindblad B, Bergqvist D. Endothelial cell seeded dacron aortobifurcated grafts: platelet deposition and long-term follow-up. Journal of Cardiovascular Surgery 1994; 35(5):425-429.

(91) Kadletz M, Magometschnigg H, Minar E, Konig G, Grabenwoger M, Grimm et al. Implantation of in vitro endothelialized polytetrafluoroethylene grafts in human beings. A preliminary report. Journal of Thoracic & Cardiovascular Surgery 1992; 104(3):736-742.

(92) Ortenwall P, Wadenvik H, Kutti J, Risberg B. Endothelial cell seeding reduces thrombogenicity of Dacron grafts in humans. Journal of Vascular Surgery 1990; 11(3):403-410.

(93) Magometschnigg H, Kadletz M, Vodrazka M, Dock W, Grimm M, Grabenwoger M et al. Prospective clinical study with in vitro endothelial cell lining of expanded polytetrafluoroethylene grafts in crural repeat reconstruction. Journal of Vascular Surgery 1992; 15:527-535.

(94) Jarrell BE, Williams SK, Stokes G, Hubbard FA, Carabasi RA, Koolpe E et al. Use of freshly isolated capillary endothelial cells for the immediate establishment of a monolayer on a vascular graft at surgery. Surgery 1986; 100(2):392-399.

(95) Tiwari A, DiSalvo C, Walesby R, Hamilton G, Seifalian AM. Mediastinal fat: a source of cells for tissue engineering of coronary artery bypass grafts. Microvasc Res 2003; 65(1):61-64.

(96) Tiwari A, Rashid ST, Salacinski H, Hamilton G, Seifalian AM. Clinical long term results of vascular prosthesis sealed with fragmented autologous adipose tissue. Artif Organs 2002; 26(2):209-210.

(97) Karube N, Soma T, Noishiki Y, Yamazaki I, Kosuge T, Ichikawa Y et al. Clinical long-term results of vascular prosthesis sealed with fragmented autologous adipose tissue. Artif Organs 2001; 25(3):218-222.

(98) Watkins MT, Sharefkin JB, Zajtchuk R, Maciag TM, D'Amore PA, Ryan US et al. Adult human saphenous vein endothelial cells: assessment of their reproductive capacity for use in endothelial seeding of vascular prostheses. J Surg Res 1984; 36(6):588-596.

(99) Sharp WV, Schmidt SP, Meerbaum SO, Pippert TR. Derivation of human microvascular endothelial cells for prosthetic vascular graft seeding. Ann Vasc Surg 1989; 3(2):104-107.

(100) Scott NA, Candal FJ, Robinson KA, Ades EW. Seeding of intracoronary stents with immortalized human microvascular endothelial cells. Am Heart J 1995; 129(5):860-866.

(101) Vici M, Pasquinelli G, Preda P, Martinelli GN, Gibellini D, Freyrie A et al. Electron microscopic and immunocytochemical profiles of human subcutaneous fat tissue microvascular endothelial cells. Ann Vasc Surg 1993; 7(6):541-548.

(102) Rupnick MA, Hubbard FA, Pratt K, Jarrell BE, Williams SK. Endothelialization of vascular prosthetic surfaces after seeding or sodding with human microvascular endothelial cells. J Vasc Surg 1989; 9(6):788-795.

(103) Pronk A, Leguit P, Hoynck van Papendrecht AA, Hagelen E, van Vroonhoven TJ, Verbrugh HA. A cobblestone cell isolated from the human omentum: the mesothelial cell; isolation, identification, and growth characteristics. In Vitro Cell Dev Biol 1993; 29A(2):127-134.

(104) Anders E, Alles JU, Delvos U, Potzsch B, Preissner KT, Muller-Berghaus G. Microvascular endothelial cells from human omental tissue: modified method for long-term cultivation and new aspects of characterization. Microvasc Res 1987; 34(2):239-249.

(105) Williams SK. Human Clinical Trials of Microvascular Endothelial Sodding. In: Zilla P, Greisler HP, editors. Tissue Engineering of Prosthetic Vascular Grafts. Austin, Texas: R.G. Landes Company, 1999: 143-147.

(106) Meerbaum SO, Sharp WV, Schmidt SP. Lower extremity revascularization with polytetrafluoroethylene grafts seeded with microvascular endothelial cells. In: Zilla P, Fasol R, Callow A, editors. Applied Cardiovascular Biology. Basel: S.Karger, 1990: 107-119.

(107) Boyer M, Townsend LE, Vogel LM, Falk J, Reitz-Vick D, Trevor KT et al. Isolation of endothelial cells and their progenitor cells from human peripheral blood. J Vasc Surg 2000; 31(1 Pt 1):181-189.

(108) Bhattacharya V, Shi Q, Ishida A, Sauvage LR, Hammond WP, Wu MH. Administration of granulocyte colony-stimulating factor enhances endothelialization and microvessel formation in small-caliber synthetic vascular grafts. J Vasc Surg 2000; 32(1):116-123.

(109) Bhattacharya V, McSweeney PA, Shi Q, Bruno B, Ishida A, Nash R et al. Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34(+) bone marrow cells. Blood 2000; 95(2):581-585.

(110) Shi Q, Bhattacharya V, Hong-De Wu M, Sauvage LR. Utilizing Granulocyte Colony-stimulating Factor to Enhance Vascular Graft Endothelialization from Circulating Blood Cells. Ann Vasc Surg 2002; 16(3):314-320.

(111) Salacinski HJ, Tiwari A, Hamilton G, Seifalian AM. Cellular engineering of vascular bypass grafts: role of chemical coatings for enhancing endothelial cell attachment. Med Biol Eng Comput 2001; 39(6):609-618.

(112) Herring MB, Gardner A, Glover J. A single-staged technique for seeding vascular grafts with autogenous endothelium. Surgery 1978; 84(4):498-504.

(113) Herring M, Smith J, Dalsing M, Glover J, Compton R, Etchberger K et al. Endothelial seeding of polytetrafluoroethylene femoral popliteal bypasses: the failure of low-density seeding to improve patency. Journal of Vascular Surgery 1994; 20(4):650-655.

(114) Rosenman JE, Kempczinski RF, Pearce WH, Silberstein EB. Kinetics of endothelial cell seeding. Journal of Vascular Surgery 1985; 2(6):778-784.

(115) Kesler KA, Herring MB, Arnold MP, Glover JL, Park HM, Helmus MN et al. Enhanced strength of endothelial attachment on polyester elastomer and polytetrafluoroethylene graft surfaces with fibronectin substrate. Journal of Vascular Surgery 1986; 3(1):58-64.

(116) Kent KC, Oshima A, Whittemore AD. Optimal seeding conditions for human endothelial cells. Annals of Vascular Surgery 1992; 6(3):258-264.

(117) Falk J, Townsend LE, Vogel LM, Boyer M, Olt S, Wease GL et al. Improved adherence of genetically modified endothelial cells to small-diameter expanded polytetrafluoroethylene grafts in a canine model. Journal of Vascular Surgery 1998; 27(5):902-908.

(118) Giudiceandrea A, Seifalian AM, Krijgsman B, Hamilton G. Effect of prolonged pulsatile shear stress in vitro on endothelial cell seeded PTFE and compliant polyurethane vascular grafts. European Journal of Vascular & Endovascular Surgery 1998; 15(2):147-154.

(119) Deutsch M, Meinhart J, Fischlein T, Preiss P, Zilla P. Clinical autologous in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients: a 9-year experience. Surgery 1999; 126(5):847-855.

(120) Laube HR, Duwe J, Rutsch W, Konertz W. Clinical experience with autologous endothelial cell-seeded polytetrafluoroethylene coronary artery bypass grafts. Journal of Thoracic & Cardiovascular Surgery 2000; 120(1):134-141.

(121) Zilla P, Deutsch M, Meinhart J. Endothelial cell transplantation. Semin Vasc Surg 1999; 12(1):52-63.

(122) Burns P, Gough S, Bradbury AW. Management of peripheral arterial disease in primary care. BMJ 2003; 326(7389):584-588.

(123) Wilson GJ, MacGregor DC, Klement P, Dereume JP, Weber BA, Binnington AG et al. The composite Corethane/Dacron vascular prosthesis. Canine in vivo evaluation of 4 mm diameter grafts with 1 year follow-up. ASAIO Trans 1991; 37(3):M475-M476.

(124) Jeschke MG, Hermanutz V, Wolf SE, Koveker GB. Polyurethane vascular prostheses decreases neointimal formation compared with expanded polytetrafluoroethylene. J Vasc Surg 1999; 29(1):168-176.

(125) Lyman DJ, Fazzio FJ, Voorhees H, Robinson G, Albo D, Jr. Compliance as a factor effecting the patency of a copolyurethane vascular graft. J Biomed Mater Res 1978; 12(3):337-345.

(126) de Cossart L, How TV, Annis D. A two year study of the performance of a small diameter polyurethane (Biomer) arterial prosthesis. J Cardiovasc Surg (Torino) 1989; 30(3):388-394.

(127) Bull PG, Denck H, Guidoin R, Gruber H. Preliminary clinical experience with polyurethane vascular prostheses in femoro-popliteal reconstruction. Eur J Vasc Surg 1992; 6(2):217-224.

(128) Allen RD, Yuill E, Nankivell BJ, Francis DM. Australian multicentre evaluation of a new polyurethane vascular access graft. Aust N Z J Surg 1996; 66(11):738-742.

(129) Ota K, Kawai T, Teraoka S, Sasaki Y, Nakagawa Y. Clinical application of a self-sealing poly(ether-urethane) graft applicable to blood access for hemodialysis. Artif Organs 1989; 13(6):498-503.

(130) Dereume JP, van Romphey A, Vincent G, Engelmann E. Femoropopliteal bypass with a compliant, composite polyurethane/Dacron graft: short-term results of a multicentre trial. Cardiovasc Surg 1993; 1(5):499-503.

(131) Nakagawa Y, Ota K, Sato Y, Fuchinoue S, Teraoka S, Agishi T. Complications in blood access for hemodialysis. Artif Organs 1994; 18(4):283-288.

(132) Brothers TE, Stanley JC, Burkel WE, Graham LM. Small-caliber polyurethane and polytetrafluoroethylene grafts: a comparative study in a canine aortoiliac model. Journal of Biomedical Materials Research 1990; 24(6):761-771.

(133) Seifalian AM, Salacinski HJ, Tiwari A, Edwards A, Bowald S, Hamilton G. In vivo biostability of a poly(carbonate-urea)urethane graft. Biomaterials 2003; 24(14):2549-2557.

(134) Tiwari A, Salacinski H, Seifalian AM, Hamilton G. New prostheses for use in bypass grafts with special emphasis on polyurethanes. Cardiovasc Surg 2002; 10(3):191-197.

(135) Salacinski HJ, Tai NR, Carson RJ, Edwards A, Hamilton G, Seifalian AM. In vitro stability of a novel compliant poly(carbonate-urea)urethane to oxidative and hydrolytic stress. J Biomed Mater Res 2002; 59(2):207-218.

(136) Stansby G, Berwanger C, Shukla N, Schmitz-Rixen T, Hamilton G. Endothelial seeding of compliant polyurethane vascular graft material. British Journal of Surgery 1994; 81(9):1286-1289.

(137) Edwards A, Carson RJ, Szycher M, Bowald S. In vitro and in vivo biodurability of a compliant microporous vascular graft. J Biomater Appl 1998; 13(1):23-45.

(138) Tai NR, Giudiceandrea A, Salacinski HJ, Seifalian AM, Hamilton G. In vivo femoropopliteal arterial wall compliance in subjects with and without lower limb vascular disease. J Vasc Surg 1999; 30(5):936-945.

(139) Tai NR, Salacinski HJ, Edwards A, Hamilton G, Seifalian AM. Compliance properties of conduits used in vascular reconstruction. British Journal of Surgery 2000; 87(11):1516-1524.

(140) Salacinski HJ, Punshon G, Krijgsman B, Hamilton G, Seifalian AM. A hybrid compliant vascular graft seeded with microvascular endothelial cells extracted from human omentum. Artif Organs 2001; 25(12):974-982.

(141) Tiwari A, Salacinski HJ, Punshon G, Hamilton G, Seifalian AM. Development of a hybrid cardiovascular graft using a tissue engineering approach. FASEB J 2002; 16(8):791-796.

(142) Langer R, Vacanti JP. Tissue engineering. Science 1993; 260(5110):920-926.

(143) Nabel EG, Plautz G, Boyce FM, Stanley JC, Nabel GJ. Recombinant gene expression in vivo within endothelial cells of the arterial wall. Science 1989; 244(4910):1342-1344.

(144) Plautz G, Nabel EG, Nabel GJ. Introduction of vascular smooth muscle cells expressing recombinant genes in vivo. Circulation 1991; 83(2):578-583.

(145) Nabel EG, Plautz G, Nabel GJ. Gene transfer into vascular cells. J Am Coll Cardiol 1991; 17(6 Suppl B):189B-194B.

(146) Kuo MD, Waugh JM, Yuksel E, Weinfeld AB, Yuksel M, Dake MD. 1998 ARRS President's Award. The potential of in vivo vascular tissue engineering for the treatment of vascular thrombosis: a preliminary report. American Roentgen Ray Society. AJR Am J Roentgenol 1998; 171(3):553-558.

(147) Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275(5302):964-967.

(148) Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999; 85(3):221-228.

(149) Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999; 5(4):434-438.

(150) Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003; 9(6):702-712.

(151) Rafii S, Meeus S, Dias S, Hattori K, Heissig B, Shmelkov S et al. Contribution of marrow-derived progenitors to vascular and cardiac regeneration. Semin Cell Dev Biol 2002; 13(1):61-67.

(152) Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003; 361(9351):45-46.

(153) Hagege AA, Carrion C, Menasche P, Vilquin JT, Duboc D, Marolleau JP et al. Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy. Lancet 2003; 361(9356):491-492.

(154) Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. Lancet 2003; 361(9351):47-49.

(155) Orlic D, Hill JM, Arai AE. Stem cells for myocardial regeneration. Circ Res 2002; 91(12):1092-1102.

(156) Forrester JS, Price MJ, Makkar RR. Stem cell repair of infarcted myocardium: an overview for clinicians. Circulation 2003; 108(9):1139-1145.

(157) Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 2002; 360(9331):427-435.

(158) Medvinsky A, Smith A. Stem cells: Fusion brings down barriers. Nature 2003; 422(6934):823-825.

(159) Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. Nature 2003; 422(6934):901-904.

(160) Williams SF, Martin DP, Horowitz DM, Peoples OP. PHA applications: addressing the price performance issue: I. Tissue engineering. Int J Biol Macromol 1999; 25(1-3):111-121.

(161) Wong AH, Waugh JM, Amabile PG, Yuksel E, Dake MD. In vivo vascular engineering: directed migration of smooth muscle cells to limit neointima. Tissue Eng 2002; 8(2):189-199.

(162) Nathan A, Nugent MA, Edelman ER. Tissue engineered perivascular endothelial cell implants regulate vascular injury. Proceedings of the National Academy of Sciences of the United States of America 1995; 92(18):8130-8134.

(163) Nugent HM, Edelman ER. Endothelial implants provide long-term control of vascular repair in a porcine model of arterial injury. J Surg Res 2001; 99(2):228-234.

(164) Nugent HM, Rogers C, Edelman ER. Endothelial implants inhibit intimal hyperplasia after porcine angioplasty. Circ Res 1999; 84(4):384-391.

(165) Epstein SE, Fuchs S, Zhou YF, Baffour R, Kornowski R. Therapeutic interventions for enhancing collateral development by administration of growth factors: basic principles, early results and potential hazards. Cardiovasc Res 2001; 49(3):532-542.

(166) Soker S, Machado M, Atala A. Systems for therapeutic angiogenesis in tissue engineering. World Journal of Urology 2000; 18(1):10-18.

(167) Donnelly R, Yeung JM. Therapeutic angiogenesis: a step forward in intermittent claudication. Lancet 2002; 359(9323):2048-2050.

(168) Lederman RJ, Mendelsohn FO, Anderson RD, Saucedo JF, Tenaglia AN, Hermiller JB et al. Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. Lancet 2002; 359(9323):2053-2058.

(169) Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ et al. The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis. Circulation 2003; 107(10):1359-1365.

(170) Rajagopalan S, Mohler ER, III, Lederman RJ, Mendelsohn FO, Saucedo JF, Goldman CK et al. Regional angiogenesis with vascular endothelial growth factor in peripheral arterial disease: a phase II randomized, double-blind, controlled study of adenoviral delivery of vascular endothelial growth factor 121 in patients with disabling intermittent claudication. Circulation 2003; 108(16):1933-1938.

(171) Rabkin E, Schoen FJ. Cardiovascular tissue engineering. Cardiovasc Pathol 2002; 11(6):305-317.

(172) Thomas AC, Campbell GR, Campbell JH. Advances in vascular tissue engineering. Cardiovasc Pathol 2003; 12(5):271-276.

(173) Burkitt HG, Young B, Heath JW. Wheater's Functional Histology: A Text and Colour Atlas. 3 ed. New York: Churchill Livingstone, 1993.

(174) Ratcliffe A. Tissue engineering of vascular grafts. Matrix Biology 2000; 19(4):353-357.

(175) Murata K, Motayama T, Kotake C. Collagen types in various layers of the human aorta and their changes with the atherosclerotic process. Atherosclerosis 1986; 60:251-262.

(176) Ziegler T, Nerem RM. Tissue engineering a blood vessel: regulation of vascular biology by mechanical stresses. J Cell Biochem 1994; 56(2):204-209.

(177) Lantz GC, Badylak SF, Hiles MC, Coffey AC, Geddes LA, Kokini K et al. Small intestinal submucosa as a vascular graft: A review. Journal of Investigative Surgery 1993; 6(3):297-310.

(178) Huynh T, Abraham G, Murray J, Brockbank K, Hagen PO, Sullivan S. Remodeling of an acellular collagen graft into a physiologically responsive neovessel. Nature Biotechnology 1999; 17(11):1083-1086.

(179) Bader A, Steinhoff G, Strobl K, Schilling T, Brandes G, Mertsching H et al. Engineering of human vascular aortic tissue based on a xenogeneic starter matrix. Transplantation 2000; 70(1):7-14.

(180) Clarke DR, Lust RM, Sun YS, Black KS, Ollerenshaw JD. Transformation of nonvascular acellular tissue matrices into durable vascular conduits. Ann Thorac Surg 2001; 71(5 Suppl):S433-S436.

(181) Conklin BS, Richter ER, Kreutziger KL, Zhong DS, Chen C. Development and evaluation of a novel decellularized vascular xenograft. Med Eng Phys 2002; 24(3):173-183.

(182) Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med 2001; 7(9):1035-1040.

(183) Kallenbach K, Leyh RG, Lefik E, Walles T, Wilhelmi M, Cebotari S et al. Guided tissue regeneration: porcine matrix does not transmit PERV. Biomaterials 2004; 25(17):3613-3620.

(184) Greisler HP. Arterial regeneration over absorbable prostheses. Arch Surg 1982; 117(11):1425-1431.

(185) Greisler HP, Ellinger J, Schwarcz TH, Golan J, Raymond RM, Kim DU. Arterial regeneration over polydioxanone prostheses in the rabbit. Arch Surg 1987; 122(6):715-721.

(186) Greisler HP, Tattersall CW, Klosak JJ, Cabusao EA, Garfield JD, Kim DU. Partially bioresorbable vascular grafts in dogs. Surgery 1991; 110(4):645-654.

(187) Yue X, van der LB, Schakenraad JM, van Oene GH, Kuit JH, Feijen J et al. Smooth muscle cell seeding in biodegradable grafts in rats: a new method to enhance the process of arterial wall regeneration. Surgery 1988; 103(2):206-212.

(188) Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R et al. Functional arteries grown in vitro. Science 1999; 284(5413):489-493.

(189) Shum-Tim D, Stock U, Hrkach J, Shinoka T, Lien J, Moses MA et al. Tissue engineering of autologous aorta using a new biodegradable polymer. Annals of Thoracic Surgery 1999; 68(6):2298-2304.

(190) Weinberg CB, Bell E. A blood vessel model constructed from collagen and cultured vascular cells. Science 1986; 231(4736):397-400.

(191) Baguneid M, Murray D, Salacinski HJ, Fuller B, Hamilton G, Walker M et al. Shear stress preconditioning and tissue engineering based paradigms for generating arterial substitutes. Biotechnol Appl Biochem 2004; 39(2):151-157.

(192) Matsuda T, Miwa H. A hybrid vascular model biomimicking the hierarchic structure of arterial wall: Neointimal stability and neoarterial regeneration process under arterial circulation. Journal of Thoracic & Cardiovascular Surgery 1995; 110(4 I):988-997.

(193) Miwa H, Matsuda T, Iida F. Development of a hierarchically structured hybrid vascular graft biomimicking natural arteries. ASAIO Journal 1993; 39(3):M273-M277.

(194) Ishibashi K, Kawazoe K, Matsuda T. Development of a hybrid vascular graft hierarchically layered three cell types. Japanese Journal of Artificial Organs 1996; 25(3):733-737.

(195) Ishibashi K, Kawazoe K, Matsuda T. Reconstruction of a hybrid vascular graft with three cell types. Japanese Journal of Artificial Organs 1995; 24(1):150-155.

(196) Ishibashi K, Matsuda T. Reconstruction of a hybrid vascular graft hierarchically layered with three cell types. ASAIO Journal 1994; 40(3):M284-M290.

(197) Miwa H, Matsuda T, Kondo K, Tani N, Fukaya Y, Morimoto M et al. Improved patency of an elastomeric vascular graft by hybridization. ASAIO Journal 1992; 38(3):M512-M515.

(198) Miwa H, Matsuda T, Tani N, Kondo K, Iida F. An in vitro endothelialized compliant vascular graft minimizes anastomotic hyperplasia. ASAIO Journal 1993; 39(3):M501-M505.

(199) Miwa H, Matsuda T. An integrated approach to the design and engineering of hybrid arterial prostheses. Journal of Vascular Surgery 1994; 19(4):658-667.

(200) Matsuda T, Akutsu T, Kira K, Matsumoto H. Development of hybrid compliant graft: rapid preparative method for reconstruction of a vascular wall. ASAIO Transactions 1989; 35(3):553-555.

(201) He H, Shirota T, Yasui H, Matsuda T. Canine endothelial progenitor cell-lined hybrid vascular graft with nonthrombogenic potential. J Thorac Cardiovasc Surg 2003; 126(2):455-464.

(202) Sparks SR, Tripathy U, Broudy A, Bergan JJ, Kumins NH, Owens EL. Small-caliber mesothelial cell-layered polytetraflouroethylene vascular grafts in New Zealand white rabbits. Ann Vasc Surg 2002; 16(1):73-76.

(203) Hirai J, Kanda K, Oka T, Matsuda T. Highly oriented, tubular hybrid vascular tissue for a low pressure circulatory system. ASAIO Journal 1994; 40(3):M383-M388.

(204) Hirai J, Matsuda T. Self-organized, tubular hybrid vascular tissue composed of vascular cells and collagen for low-pressure-loaded venous system. Cell Transplantation 1995; 4(6):597-608.

(205) Hirai J, Matsuda T. Venous reconstruction using hybrid vascular tissue composed of vascular cells and collagen: Tissue regeneration process. Cell Transplantation 1996; 5(1):93-105.

(206) Kobashi T, Matsuda T. Fabrication of compliant hybrid grafts supported with elastomeric meshes. Cell Transplantation 1999; 8(5):477-488.

(207) Kobashi T, Matsuda T. Fabrication of branched hybrid vascular prostheses. Tissue Engineering 1999; 5(6):515-524.

(208) He H, Matsuda T. Arterial replacement with compliant hierarchic hybrid vascular graft: biomechanical adaptation and failure. Tissue Eng 2002; 8(2):213-224.

(209) He H, Matsuda T. Newly designed compliant hierarchic hybrid vascular graft wrapped with microprocessed elastomeric film--II: Morphogenesis and compliance change upon implantation. Cell Transplant 2002; 11(1):75-87.

(210) Berglund JD, Mohseni MM, Nerem RM, Sambanis A. A biological hybrid model for collagen-based tissue engineered vascular constructs. Biomaterials 2003; 24(7):1241-1254.

(211) L'Heureux N, Pâquet S, Labbé R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel [see comments]. FASEB Journal 1998; 12(1):47-56.

(212) Campbell JH, Efendy JL, Campbell GR. Novel vascular graft grown within recipient's own peritoneal cavity. Circ Res 1999; 85(12):1173-1178.

(213) Tsukagoshi T, Yenidunya MO, Sasaki E, Suse T, Hosaka Y. Experimental vascular graft using small-caliber fascia-wrapped fibrocollagenous tube: short-term evaluation. Journal of Reconstructive Microsurgery 1999; 15(2):127-131.

(214) Budd JS, Bell PR, James RF. Attachment of indium-111 labelled endothelial cells to pretreated polytetrafluoroethylene vascular grafts. Br J Surg 1989; 76(12):1259-1261.

(215) Thomson GJ, Vohra R, Walker MG. Cell seeding for small diameter ePTFE vascular grafts: a comparison between adult human endothelial and mesothelial cells. Annals of Vascular Surgery 1989; 3(2):140-145.

(216) Anderson JS, Price TM, Hanson SR, Harker LA. In vitro endothelialization of small-caliber vascular grafts. Surgery 1987; 101(5):577-586.

(217) Hess F, Jerusalem R, Reijnders O, Jerusalem C, Steeghs S, Braun B et al. Seeding of enzymatically derived and subcultivated canine endothelial cells on fibrous polyurethane vascular prostheses. Biomaterials 1992; 13(10):657-663.

(218) Williams SK, Jarrell BE, Friend L, Radomski JS, Carabasi RA, Koolpe et al. Adult human endothelial cell compatibility with prosthetic graft material. Journal of Surgical Research 1985; 38(6):618-629.

(219) Zhu Y, Gao C, He T, Shen J. Endothelium regeneration on luminal surface of polyurethane vascular scaffold modified with diamine and covalently grafted with gelatin. Biomaterials 2004; 25(3):423-430.

(220) Hayward IP, Bridle KR, Campbell GR, Underwood PA, Campbell JH. Effect of extracellular matrix proteins on vascular smooth muscle cell phenotype. Cell Biol Int 1995; 19(10):839-846.

(221) Tanahashi K, Mikos AG. Protein adsorption and smooth muscle cell adhesion on biodegradable agmatine-modified poly(propylene fumarate-co-ethylene glycol) hydrogels. J Biomed Mater Res 2003; 67A(2):448-457.

(222) Tanahashi K, Mikos AG. Cell adhesion on poly(propylene fumarate-co-ethylene glycol) hydrogels. J Biomed Mater Res 2002; 62(4):558-566.

(223) Vohra RK, Thomson GJ, Sharma H, Carr HM, Walker MG. Effects of shear stress on endothelial cell monolayers on expanded polytetrafluoroethylene (ePTFE) grafts using preclot and fibronectin matrices. Eur J Vasc Surg 1990; 4(1):33-41.

(224) Stansby G, Shukla N, Fuller B, Hamilton G. Seeding of human microvascular endothelial cells onto polytetrafluoroethylene graft material [see comments]. British Journal of Surgery 1991; 78(10):1189-1192.

(225) Vohra R, Thomson GJ, Carr HM, Sharma H, Walker MG. Comparison of different vascular prostheses and matrices in relation to endothelial seeding. British Journal of Surgery 1991; 78(4):417-420.

(226) Gosselin C, Vorp DA, Warty V, Severyn DA, Dick EK, Borovetz HS et al. ePTFE coating with fibrin glue, FGF-1, and heparin: effect on retention of seeded endothelial cells. Journal of Surgical Research 1996; 60(2):327-332.

(227) Dalsing MC, Kevorkian M, Raper B, Nixon C, Lalka SG, Cikrit DF et al. An experimental collagen-impregnated Dacron graft: potential for endothelial seeding. Annals of Vascular Surgery 1989; 3(2):127-133.

(228) Walpoth BH, Rogulenko R, Tikhvinskaia E, Gogolewski S, Schaffner T, Hess OM et al. Improvement of patency rate in heparin-coated small synthetic vascular grafts. Circulation 1998; 98(19 Suppl):II319-II323.

(229) Ozaki CK, Phaneuf MD, Hong SL, Quist WC, LoGerfo FW. Glycoconjugate mediated endothelial cell adhesion to Dacron polyester film. J Vasc Surg 1993; 18(3):486-494.

(230) Mann BK, Tsai AT, Scott-Burden T, West JL. Modification of surfaces with cell adhesion peptides alters extracellular matrix deposition. Biomaterials 1999; 20(23-24):2281-2286.

(231) Walluscheck KP, Steinhoff G, Kelm S, Haverich A. Improved endothelial cell attachment on ePTFE vascular grafts pretreated with synthetic RGD-containing peptides. European Journal of Vascular & Endovascular Surgery 1996; 12(3):321-330.

(232) Walluscheck KP, Steinhoff G, Haverich A. Endothelial cell seeding of de-endothelialised human arteries: improvement by adhesion molecule induction

and flow-seeding technology. European Journal of Vascular & Endovascular Surgery 1996; 12(1):46-53.

(233) Mann BK, West JL. Cell adhesion peptides alter smooth muscle cell adhesion, proliferation, migration, and matrix protein synthesis on modified surfaces and in polymer scaffolds. J Biomed Mater Res 2002; 60(1):86-93.

(234) Hsu SH, Sun SH, Chen DC. Improved retention of endothelial cells seeded on polyurethane small-diameter vascular grafts modified by a recombinant RGD-containing protein. Artif Organs 2003; 27(12):1068-1078.

(235) Moghaddam MJ, Matsuda T. Development of a 3-D artificial extracellular matrix. Design concept and artificial vascular media. ASAIO Trans 1991; 37(3):M437-M438.

(236) Massia SP, Stark J. Immobilized RGD peptides on surface-grafted dextran promote biospecific cell attachment. J Biomed Mater Res 2001; 56(3):390-399.

(237) Ruoslahti E. RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol 1996; 12:697-715.

(238) L'Heureux N, Stoclet JC, Auger FA, Lagaud GJ, Germain L, Andriantsitohaina R. A human tissue-engineered vascular media: a new model for pharmacological studies of contractile responses. FASEB J 2001; 15(2):515-524.

(239) Shin'oka T, Imai Y, Ikada Y. Transplantation of a tissue-engineered pulmonary artery. N Engl J Med 2001; 344(7):532-533.

(240) Matsumura G, Hibino N, Ikada Y, Kurosawa H, Shin'oka T. Successful application of tissue engineered vascular autografts: clinical experience. Biomaterials 2003; 24(13):2303-2308.

(241) Isomatsu Y, Shin'oka T, Matsumura G, Hibino N, Konuma T, Nagatsu M et al. Extracardiac total cavopulmonary connection using a tissue-engineered graft. J Thorac Cardiovasc Surg 2003; 126(6):1958-1962.

(242) Campbell JH, Campbell GR. Culture techniques and their applications to studies of vascular smooth muscle. Clin Sci (Lond) 1993; 85(5):501-513.

(243) Thyberg J. Differentiated properties and proliferation of arterial smooth muscle cells in culture. Int Rev Cytol 1996; 169:183-265.

(244) Chamley-Campbell J, Campbell GR, Ross R. The smooth muscle cell in culture. Physiol Rev 1979; 59(1):1-61.

(245) Ross R. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. Journal of Cell Biology 1971; 50(1):172-186.

(246) Chamley JH, Campbell GR, McConnell JD, Groschel-Stewart U. Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in

primary culture and in subculture. Cell & Tissue Research 1977; 177(4):503-522.

(247) Gimbrone MA, Jr., Cotran RS. Human vascular smooth muscle in culture. Growth and ultrastructure. Laboratory Investigation 1975; 33(1):16-27.

(248) Thyberg J, Nilsson J, Palmberg L, Sjölund M. Adult human arterial smooth muscle cells in primary culture. Modulation from contractile to synthetic phenotype. Cell Tissue Research 1985; 239:69-74.

(249) Berk BC. Vascular smooth muscle growth: autocrine growth mechanisms. Physiol Rev 2001; 81(3):999-1030.

(250) Chamley JH, Campbell GR, Burnstock G. Dedifferentiation, redifferentiation and bundle formation of smooth muscle cells in tissue culture: the influence of cell number and nerve fibres. Journal of Embryology & Experimental Morphology 1974; 32(2):297-323.

(251) Chamley-Campbell JH, Campbell GR. What controls smooth muscle phenotype? Atherosclerosis 1981; 40:347-357.

(252) Chamley JH, Campbell GR. Mitosis of contractile smooth muscle cells in tissue culture. Experimental Cell Research 1974; 84(1):105-110.

(253) Schwartz E, Bienkowski RS, Coltoff-Schiller B, Goldfischer S, Blumenfeld OO. Changes in the components of extracellular matrix and in growth properties of cultured aortic smooth muscle cells upon ascorbate feeding. J Cell Biol 1982; 92(2):462-470.

(254) Button M, Barden G, Fuller B, Hamilton G. Optimized extraction of smooth muscle cells from human vein for use in a tissue engineered vascular graft. Cardiovascular Pathology 11[1], 39. 2002.
Ref Type: Abstract

(255) Rashid ST, Salacinski HJ, Button MJ, Fuller B, Hamilton G, Seifalian AM. Cellular Engineering of Conduits for Coronary and Lower Limb Bypass Surgery: Role of Cell Attachment Peptides and Pre-conditioning in Optimising Smooth Muscle Cells (SMC) Adherence to Compliant Poly(carbonate-urea)urethane (MyoLink trade mark) Scaffolds. Eur J Vasc Endovasc Surg 2004; 27(6):608-616.

(256) Graham LM, Vinter DW, Ford JW, Kahn RH, Burkel WE, Stanley JC. Cultured autogenous endothelial cell seeding of prosthetic vascular grafts. Surgical Forum 1979; 30:204-206.

(257) Mansfield PB, Wechezak AR, Sauvage LR. Preventing thrombus on artifical vascular surfaces: true endothelial cell linings. Trans Am Soc Artif Intern Organs 1975; 21:264-272.

(258) Noishiki Y, Yamane Y, Tomizawa Y, Okoshi T, Satoh S, Wildevuur CR. Endothelialization of vascular prostheses by transplantation of venous tissue fragments. ASAIO Trans 1990; 36(3):M346-M348.

(259) Herring M, Dilley R, Cullison T, Gardner A, Glover J. Seeding endothelium on canine arterial prostheses--the size of the inoculum. Journal of Surgical Research 1980; 28(1):35-38.

(260) Bourke BM, Roche WR, Appleberg M. Endothelial cell harvest for seeding vascular prostheses: the influence of technique on cell function, viability, and number. J Vasc Surg 1986; 4(3):257-263.

(261) Zilla P, Deutsch M, Meinhart J, Puschmann R, Eberl T, Minar E et al. Clinical in vitro endothelialization of femoropopliteal bypass grafts: an actuarial follow-up over three years. Journal of Vascular Surgery 1994; 19(3):540-548.

(262) Scoumanne A, Kalamati T, Moss J, Powell JT, Gosling M, Carey N. Generation and characterisation of human saphenous vein endothelial cell lines. Atherosclerosis 2002; 160(1):59-67.

(263) Edwards A, Carson RJ, Bowald S, Quist WC. Development of a microporous compliant small bore vascular graft. J Biomater Appl 1995; 10(2):171-187.

(264) Hynes RO. Fibronectins. New York: Springer, 1990.

(265) Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 1984; 309(5963):30-33.

(266) LeBaron RG, Athanasiou KA. Extracellular matrix cell adhesion peptides: functional applications in orthopedic materials. Tissue Eng 2000; 6(2):85-103.

(267) Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials 2003; 24(24):4385-4415.

(268) Moiseeva EP. Adhesion receptors of vascular smooth muscle cells and their functions. Cardiovasc Res 2001; 52(3):372-386.

(269) Braun M, Pietsch P, Schror K, Baumann G, Felix SB. Cellular adhesion molecules on vascular smooth muscle cells. Cardiovasc Res 1999; 41(2):395-401.

(270) Carey DJ. Syndecans: multifunctional cell-surface co-receptors. Biochem J 1997; 327 (Pt 1):1-16.

(271) Couchman JR, Woods A. Syndecans, signaling, and cell adhesion. J Cell Biochem 1996; 61(4):578-584.

(272) Ruegg C, Mariotti A. Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. Cell Mol Life Sci 2003; 60(6):1135-1157.

(273) Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Geometric control of cell life and death. Science 1997; 276(5317):1425-1428.

(274) Graf J, Iwamoto Y, Sasaki M, Martin GR, Kleinman HK, Robey FA et al. Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding. Cell 1987; 48(6):989-996.

(275) Rubin BG, McGraw DJ, Sicard GA, Santoro SA. New RGD analogue inhibits human platelet adhesion and aggregation and eliminates platelet deposition on canine vascular grafts. J Vasc Surg 1992; 15(4):683-691.

(276) Ghandehari H, Sharan R, Rubas W, Killing WM. Molecular modeling of arginine-glycine-aspartic acid (RGD) analogs: relevance to transepithelial transport. J Pharm Pharm Sci 2001; 4(1):32-41.

(277) Bowlin GL, Rittgers SE. Electrostatic endothelial cell seeding technique for small-diameter (<6 mm) vascular prostheses: feasibility testing. Cell Transplantation 1997; 6(6):623-629.

(278) Bowlin GL, Rittgers SE, Milsted A, Schmidt SP. In vitro evaluation of electrostatic endothelial cell transplantation onto 4 mm interior diameter expanded polytetrafluoroethylene grafts. Journal of Vascular Surgery 1998; 27(3):504-511.

(279) Salacinski HJ, Tai NR, Punshon G, Giudiceandrea A, Hamilton G, Seifalian AM. Optimal endothelialisation of a new compliant Poly(Carbonate-Urea)Urethane vascular graft with effect of physiological shear stress [In Process Citation]. Eur J Vasc Endovasc Surg 2000; 20(4):342-352.

(280) Stansby G, Shukla N, Berwanger C, Seifalian AM, Fuller B, Hamilton G. ^{111}Indium labeling of endothelial cells: potential problems when applied to studies of vascular graft seeding. International J Angiology 1994; 3:86-89.

(281) Seifalian AM, Salacinski HJ, Punshon G, Krijgsman B, Hamilton G. A new technique for measuring the cell growth and metabolism of endothelial cells seeded on vascular prostheses. J Biomed Mater Res 2001; 55(4):637-644.

(282) Ramamurthi A, Vesely I. Smooth muscle cell adhesion on crosslinked hyaluronan gels. J Biomed Mater Res 2002; 60(1):195-205.

(283) Koyano T, Minoura N, Nagura M, Kobayashi K. Attachment and growth of cultured fibroblast cells on PVA/chitosan- blended hydrogels. J Biomed Mater Res 1998; 39(3):486-490.

(284) Krijgsman B, Seifalian AM, Salacinski HJ, Tai NR, Punshon G, Fuller BJ et al. An Assessment of Covalent Grafting of RGD Peptides to the Surface of a Compliant Poly(Carbonate-Urea)Urethane Vascular Conduit versus Conventional Biological Coatings: Its Role in Enhancing Cellular Retention. Tissue Eng 2002; 8(4):673-680.

(285) Seifalian AM, Tiwari A, Hamilton G, Salacinski HJ. Improving the clinical patency of prosthetic vascular and coronary bypass grafts: the role of seeding and tissue engineering. Artif Organs 2002; 26(4):307-320.

(286) Stephens DJ, Allan VJ. Light microscopy techniques for live cell imaging. Science 2003; 300(5616):82-86.

(287) Lippincott-Schwartz J, Patterson GH. Development and use of fluorescent protein markers in living cells. Science 2003; 300(5616):87-91.

(288) Afting M, Stock UA, Nasseri B, Pomerantseva I, Seed B, Vacanti JP. Efficient and stable retroviral transfection of ovine endothelial cells with green fluorescent protein for cardiovascular tissue engineering. Tissue Eng 2003; 9(1):137-141.

(289) Palu G, Parolin C, Takeuchi Y, Pizzato M. Progress with retroviral gene vectors. Rev Med Virol 2000; 10(3):185-202.

(290) Hicks GG, Chen J, Ruley HE. Production and Use of Retroviruses. In: Ravid K, Freshney RI, editors. DNA Transfer to Cultured Cells. Wiley-Liss Inc., 1998: 1-26.

(291) Kanda K, Matsuda T. Mechanical stress-induced orientation and ultrastructural change of smooth muscle cells cultured in three-dimensional collagen lattices. Cell Transplantation 1994; 3(6):481-492.

(292) Mills I, Sumpio BE. Mechanical Forces and Cell Differentiation. In: Zilla P, Greisler HP, editors. Tissue Engineering of Vascular Prosthetic Grafts. Austin, Texas: R.G. Landes, 1999: 425-438.

(293) Davies PF. Flow-mediated endothelial mechanotransduction. Physiol Rev 1995; 75(3):519-560.

(294) Shigematsu K, Yasuhara H, Shigematsu H, Muto T. Direct and indirect effects of pulsatile shear stress on the smooth muscle cell. Inter Angio 2000; 19(1):39-46.

(295) Kraiss LW, Geary RL, Mattsson EJ, Vergel S, Au YP, Clowes AW. Acute reductions in blood flow and shear stress induce platelet-derived growth factor-A expression in baboon prosthetic grafts. Circ Res 1996; 79(1):45-53.

(296) Kohler TR, Kirkman TR, Kraiss LW, Zierler BK, Clowes AW. Increased blood flow inhibits neointimal hyperplasia in endothelialized vascular grafts. Circ Res 1991; 69(6):1557-1565.

(297) Geary RL, Kohler TR, Vergel S, Kirkman TR, Clowes AW. Time course of flow-induced smooth muscle cell proliferation and intimal thickening in endothelialized baboon vascular grafts. Circ Res 1994; 74(1):14-23.

(298) Lee AA, Graham DA, Dela CS, Ratcliffe A, Karlon WJ. Fluid shear stress-induced alignment of cultured vascular smooth muscle cells. J Biomech Eng 2002; 124(1):37-43.

(299) Sterpetti AV, Cucina A, D'Angelo LS, Cardillo B, Cavallaro A. Response of arterial smooth muscle cells to laminar flow. J Cardiovasc Surg (Torino) 1992; 33(5):619-624.

(300) Buck RC. Behaviour of vascular smooth muscle cells during repeated stretching of the substratum in vitro. Atherosclerosis 1983; 46:217-233.

(301) Dartsch PC, Hammerle H. Orientation response of arterial smooth muscle cells to mechanical stimulation. European Journal of Cell Biology 1986; 41:339.

(302) Dartsch PC, Hammerle H, Betz E. Orientation of cultured arterial smooth muscle cells growing on cyclically stretched substrates. Acta Anatomica 1986; 125(2):108-113.

(303) Kanda K, Matsuda T. Behavior of arterial wall cells cultured on periodically stretched substrates. Cell Transplantation 1993; 2(6):475-484.

(304) Birukov KG, Shirinsky VP, Stepanova OV, Tkachuk VA, Hahn AW, Resink TJ et al. Stretch affects phenotype and proliferation of vascular smooth muscle cells. Mol Cell Biochem 1995; 144(2):131-139.

(305) Kolpakov V, Rekhter MD, Gordon D, Wang WH, Kulik TJ. Effect of mechanical forces on growth and matrix protein synthesis in the in vitro pulmonary artery. Analysis of the role of individual cell types. Circ Res 1995; 77(4):823-831.

(306) Nikolovski J, Kim BS, Mooney DJ. Cyclic strain inhibits switching of smooth muscle cells to an osteoblast-like phenotype. FASEB J 2003; 17(3):455-457.

(307) Hamilton DW, Maul TM, Vorp DA. Characterization of the response of bone marrow-derived progenitor cells to cyclic strain: implications for vascular tissue-engineering applications. Tissue Eng 2004; 10(3-4):361-369.

(308) Leung DY, Glagov S, Mathews MB. Cyclic stretching stimulates synthesis of matrix components by arterial smooth muscle cells in vitro. Science 1976; 191(4226):475-477.

(309) Kim BS, Nikolovski J, Bonadio J, Mooney DJ. Cyclic mechanical strain regulates the development of engineered smooth muscle tissue. Nat Biotechnol 1999; 17(10):979-983.

(310) Niklason LE, Abbott W, Gao J, Klagges B, Hirschi KK, Ulubayram K et al. Morphologic and mechanical characteristics of engineered bovine arteries. J Vasc Surg 2001; 33(3):628-638.

(311) Cardiopulmonary bypass / pumps . 1998.
Ref Type: Internet Communication

(312) Thompson CA. A Novel Pulsatile, Laminar Flow Bioreactor for the Development of Tissue-Engineered Vascular Structures. Tissue Eng 2002; 8(6):1083-1088.

(313) Hoerstrup SP, Sodian R, Sperling JS, Vacanti JP, Mayer JE, Jr. New pulsatile bioreactor for in vitro formation of tissue engineered heart valves. Tissue Eng 2000; 6(1):75-79.

(314) Sodian R, Lemke T, Fritsche C, Hoerstrup SP, Fu P, Potapov EV et al. Tissue-engineering bioreactors: a new combined cell-seeding and perfusion system for vascular tissue engineering. Tissue Eng 2002; 8(5):863-870.

(315) Sodian R, Lemke T, Loebe M, Hoerstrup SP, Potapov EV, Hausmann H et al. New pulsatile bioreactor for fabrication of tissue-engineered patches. J Biomed Mater Res 2001; 58(4):401-405.

(316) Hoerstrup SP, Zund G, Sodian R, Schnell AM, Grunenfelder J, Turina MI. Tissue engineering of small caliber vascular grafts. Eur J Cardiothorac Surg 2001; 20(1):164-169.

(317) Bancroft GN, Sikavitsas VI, Mikos AG. Design of a flow perfusion bioreactor system for bone tissue-engineering applications. Tissue Eng 2003; 9(3):549-554.

(318) Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R et al. Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization. Biotechnol Bioeng 1999; 64(5):580-589.

(319) Jockenhoevel S, Zund G, Hoerstrup SP, Schnell A, Turina M. Cardiovascular tissue engineering: a new laminar flow chamber for in vitro improvement of mechanical tissue properties. ASAIO J 2002; 48(1):8-11.

(320) McCulloch AD, Harris AB, Sarraf CE, Eastwood M. New multi-cue bioreactor for tissue engineering of tubular cardiovascular samples under physiological conditions. Tissue Eng 2004; 10(3-4):565-573.

(321) Mironov V, Kasyanov V, McAllister K, Oliver S, Sistino J, Markwald R. Perfusion bioreactor for vascular tissue engineering with capacities for longitudinal stretch. J Craniofac Surg 2003; 14(3):340-347.

(322) Clerin V, Nichol JW, Petko M, Myung RJ, Gaynor JW, Gooch KJ. Tissue engineering of arteries by directed remodeling of intact arterial segments. Tissue Eng 2003; 9(3):461-472.

(323) Passerini AG, Milsted A, Rittgers SE. Shear stress magnitude and directionality modulate growth factor gene expression in preconditioned vascular endothelial cells. J Vasc Surg 2003; 37(1):182-190.

(324) Solan A, Mitchell S, Moses M, Niklason L. Effect of pulse rate on collagen deposition in the tissue-engineered blood vessel. Tissue Eng 2003; 9(4):579-586.

(325) Dunkern TR, Paulitschke M, Meyer R, Buttemeyer R, Hetzer R, Burmester G et al. A novel perfusion system for the endothelialisation of PTFE grafts under defined flow. European Journal of Vascular & Endovascular Surgery 1999; 18(2):105-110.

(326) Dardik A, Liu A, Ballermann BJ. Chronic in vitro shear stress stimulates endothelial cell retention on prosthetic vascular grafts and reduces subsequent

in vivo neointimal thickness. Journal of Vascular Surgery 1999; 29(1):157-167.

(327) Conklin BS, Surowiec SM, Lin PH, Chen C. A simple physiologic pulsatile perfusion system for the study of intact vascular tissue. Med Eng Phys 2000; 22(6):441-449.

(328) Surowiec SM, Conklin BS, Li JS, Lin PH, Weiss VJ, Lumsden AB et al. A new perfusion culture system used to study human vein. J Surg Res 2000; 88(1):34-41.

(329) Giudiceandrea A, Salacinski H, Tai N, Punshon G, Hamilton G, Seifalian A. Development and evaluation of an ideal flow circuit: assessing the dynamic behavior of endothelial cell seeded grafts. J Artif Organs 2000; 3:16-24.

(330) Yu H, Wang Y, Eton D, Rowe VL, Terramani TT, Cramer DV et al. Dual cell seeding and the use of zymogen tissue plasminogen activator to improve cell retention on polytetrafluoroethylene grafts. J Vasc Surg 2001; 34(2):337-343.

(331) Yu H, Dai W, Yang Z, Kirkman P, Weaver FA, Eton D et al. Smooth muscle cells improve endothelial cell retention on polytetrafluoroethylene grafts in vivo. J Vasc Surg 2003; 38(3):557-563.

(332) Ruiz-Torres A, Gimeno A, Melon J, Mendez L, Munoz FJ, Macia M. Age-related loss of proliferative activity of human vascular smooth muscle cells in culture. Mechanisms of Ageing & Development 1999; 110(1-2):49-55.

(333) Bierman EL. The effect of donor age on the in vitro life span of cultured human arterial smooth-muscle cells. In Vitro 1978; 14(11):951-955.

(334) Smith JR, Lincoln DW. Aging of cells in culture. Int Rev Cytol 1984; 89:151-177.

(335) Moczar M, Ouzilou J, Courtois Y, Robert L. Age dependence of the biosynthesis of intercellular matrix macromolecules of rabbit aorta in organ culture and cell culture. Gerontology 1976; 22(6):461-472.

(336) Ogle BM, Mooradian DL. The role of vascular smooth muscle cell integrins in the compaction and mechanical strengthening of a tissue-engineered blood vessel. Tissue Engineering 1999; 5(4):387-402.

(337) Nikolovski J, Mooney DJ Smooth muscle cell adhesion to tissue engineering scaffolds. Biomaterials 2000; 21(20):2025-2032.

(338) Buckley CD, Pilling D, Henriquez NV, Parsonage G, Threlfall K, Scheel-Toellner D et al. RGD peptides induce apoptosis by direct caspase-3 activation. Nature 1999; 397(6719):534-539.

(339) Hern DL, Hubbell JA. Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing. J Biomed Mater Res 1998; 39(2):266-276.

(340) Maheshwari G, Brown G, Lauffenburger DA, Wells A, Griffith LG. Cell adhesion and motility depend on nanoscale RGD clustering. J Cell Sci 2000; 113 (Pt 10):1677-1686.

(341) Brandley BK, Schnaar RL. Covalent attachment of an Arg-Gly-Asp sequence peptide to derivatizable polyacrylamide surfaces: support of fibroblast adhesion and long-term growth. Anal Biochem 1988; 172(1):270-278.

(342) Jeschke B, Meyer J, Jonczyk A, Kessler H, Adamietz P, Meenen NM et al. RGD-peptides for tissue engineering of articular cartilage. Biomaterials 2002; 23(16):3455-3463.

(343) Hirano Y, Okuno M, Hayashi T, Goto K, Nakajima A. Cell-attachment activities of surface immobilized oligopeptides RGD, RGDS, RGDV, RGDT, and YIGSR toward five cell lines. J Biomater Sci Polym Ed 1993; 4(3):235-243.

(344) Irvine DJ, Mayes AM, Griffith LG. Nanoscale clustering of RGD peptides at surfaces using Comb polymers. 1. Synthesis and characterization of Comb thin films. Biomacromolecules 2001; 2(1):85-94.

(345) Margel S, Vogler EA, Firment L, Watt T, Haynie S, Sogah DY. Peptide, protein, and cellular interactions with self-assembled monolayer model surfaces. J Biomed Mater Res 1993; 27(12):1463-1476.

(346) Chinn JA, Sauter JA, Phillips RE, Jr., Kao WJ, Anderson JM, Hanson SR et al. Blood and tissue compatibility of modified polyester: thrombosis, inflammation, and healing. J Biomed Mater Res 1998; 39(1):130-140.

(347) Sharefkin JB, Lather C, Smith M, Rich NM. Endothelial cell labeling with indium-111-oxine as a marker of cell attachment to bioprosthetic surfaces. J Biomed Mater Res 1983; 17(2):345-357.

(348) Patterson RB, Mayfield G, Silberstein EB, Kempczinski RF. The potential unreliability of indium 111 oxine labeling in studies of endothelial cell kinetics. J Vasc Surg 1989; 10(6):650-655.

(349) Pratt KJ, Jarrell BE, Williams SK, Carabasi RA, Rupnick MA, Hubbard FA. Kinetics of endothelial cell-surface attachment forces. J Vasc Surg 1988; 7(4):591-599.

(350) Stupack DG, Puente XS, Boutsaboualoy S, Storgard CM, Cheresh DA. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. J Cell Biol 2001; 155(3):459-470.

(351) Zeltinger J, Sherwood JK, Graham DA, Mueller R, Griffith LG. Effect of pore size and void fraction on cellular adhesion, proliferation, and matrix deposition. Tissue Eng 2001; 7(5):557-572.

(352) Honig MG, Hume RI. Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. J Cell Biol 1986; 103(1):171-187.

(353) Kanda K, Matsuda T, Oka T. Two-dimensional orientational response of smooth muscle cells to cyclic stretching. ASAIO Journal 1992; 38(3):M382-M385.

(354) Kanda K, Matsuda T. In vitro reconstruction of hybrid arterial media with molecular and cellular orientations. Cell Transplantation 1994; 3(6):537-545.

(355) Ott MJ, Ballermann BJ. Shear stress-conditioned, endothelial cell-seeded vascular grafts: improved cell adherence in response to in vitro shear stress. Surgery 1995; 117(3):334-339.

(356) Ballermann BJ, Ott MJ. Adhesion and differentiation of endothelial cells by exposure to chronic shear stress: a vascular graft model. Blood Purif 1995; 13(3-4):125-134.

(357) Fisher AB, Chien S, Barakat AI, Nerem RM. Endothelial cellular response to altered shear stress. Am J Physiol Lung Cell Mol Physiol 2001; 281(3):L529-L533.

(358) Wu MH, Kouchi Y, Onuki Y, Shi Q, Yoshida H, Kaplan S et al. Effect of differential shear stress on platelet aggregation, surface thrombosis, and endothelialization of bilateral carotid-femoral grafts in the dog. J Vasc Surg 1995; 22(4):382-390.

(359) Nerem RM. Tissue engineering a blood vessel substitute: the role of biomechanics. Yonsei Med J 2000; 41(6):735-739.

(360) Seliktar D, Black RA, Vito RP, Nerem RM. Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. Ann Biomed Eng 2000; 28(4):351-362.

(361) Stock UA, Vacanti JP. Cardiovascular physiology during fetal development and implications for tissue engineering. Tissue Engineering 2001; 7(1):1-7.

(362) Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000; 95(10):3106-3112.

(363) Simper D, Stalboerger PG, Panetta CJ, Wang S, Caplice NM. Smooth muscle progenitor cells in human blood. Circulation 2002; 106(10):1199-1204.

(364) Kidane AG, Salacinski HJ, Punshon G, Ramesh B, Srai KS, Seifalian AM. Synthesis and evaluation of amphiphilic RGD derivatives: uses for solvent casting in polymers and tissue engineering applications. Med Biol Eng Comput 2003; 41(6):740-745.

(365) Rashid ST, Salacinski HJ, Hamilton G, Seifalian AM. The use of animal models in developing the discipline of cardiovascular tissue engineering: a review. Biomaterials 2004; 25(9):1627-1637.

(366) Wolf S, Werthessen NT. Dynamics of Arterial Flow. New York: Plenum Press, 1978.